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Growth of *Neocallimastix* sp. Strain R1 on Italian Ryegrass Hay: Removal of Neutral Sugars from Plant Cell Walls

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The anaerobic fungus *Neocallimastix* sp. strain R1 was grown for up to 5 days on a medium containing autoclaved Italian ryegrass hay as the carbon source. Culture supernatants and digested cell walls were harvested at 12-h intervals. Supernatants were analyzed for the fermentation products formate and acetate, and residual cell walls were analyzed for dry-matter and neutral-sugar losses. Fungal growth was accompanied by the digestion of plant cell walls and the accumulation of fermentation products in culture media. Dry-matter losses were accounted for by removal of four major neutral sugars (arabinose, galactose, glucose, and xylose) from the plant cell walls. First-order reaction kinetics could be used to describe the loss of each sugar. All cell wall sugars, including arabinose and galactose, which are not fermented by *Neocallimastix* sp. strain R1 were removed simultaneously. Although the rates of removal of individual sugars were similar, there were significant differences in their extents of removal: the extent of removal of arabinose exceeded that of the other three sugars, and xylose was the least digestible. This study provides the first account of simultaneous (nonpreferential) removal of neutral sugars from plant cell walls by an anaerobic fungus. Although in vitro techniques were used, these results indicate a potentially significant role for the anaerobic fungi as fiber digesters in the rumen.

Until the mid-1970s, it was assumed that specific groups of anaerobic bacteria and, perhaps to a lesser extent, protozoa were responsible for the degradation of plant fiber in the rumen (12). However, this assumption was challenged by Orpin (22), who identified the anaerobic fungi, and by Bauchop (1, 2), who demonstrated their ability to grow on fibrous plant substrates in the rumen. Since the original communication of Orpin (22), anaerobic fungi have been found in the digestive tracts of a variety of animals, and they are generally recognized as normal members of the gut microflora, particularly in animals fed highly fibrous diets (1, 2). In addition to their presence in the rumen, anaerobic fungi have been isolated from the horse cecum (25), the feces and saliva of sheep (15), and the feces of 11 other ruminant and monogastric herbivores from a zoological garden (A. Milne, M. K. Theodorou, M. G. C. Jordan, C. King-Spooner, and A. P. J. Trinci, Exp. Mycol., in press).

The life cycle of anaerobic fungi consists of two stages, in which a motile flagellate phase (the zoospore) alternates with a nonmotile vegetative phase (the fungal thallus). When fully developed, the thallus consists of one (monocentric) or more (polycentric) zoosporangia supported by a system of branched, tapering rhizoids (14, 22). These rhizoids penetrate plant substrates for anchorage and to obtain nutrients for growth (11, 23, 31). When coupled with the ability to produce cell-wall-degrading enzymes (10, 16, 33), the anaerobic fungi are able to coexist with bacteria and protozoa in the rumen in a highly competitive microbial ecosystem.

Comparatively little is known about the precise role and overall contribution of anaerobic fungi to the degradation of fibrous plant substrates in the rumen. This is largely because of the difficulty of measuring fungal biomass and activity in a mixed population ecosystem. Bauchop (2) has proposed that the anaerobic fungi are initial colonizers of fibrous plant substrates in the rumen. It has also been suggested that fiber colonized by anaerobic fungi may be more accessible for secondary invasion by other ruminal microorganisms (31). However, although fibrolytic activity has been well documented (10, 16, 33), little information is available concerning the manner in which fiber is degraded by these organisms. In the present research, the anaerobic rumen fungus *Neocallimastix* sp. strain R1 (14) was used, together with in vitro techniques involving growth on an autoclaved substrate, to determine the ability of anaerobic fungi to degrade structural polysaccharides in plant cell walls.

(A preliminary report of part of this work has been presented previously [30].)

MATERIALS AND METHODS

Organism and culture conditions. *Neocallimastix* sp. strain R1 is an anaerobic fungus which was isolated from the rumens of sheep (18) and is the subject of previous publications (14–17). Since the original communication (18), cultures have been obtained from individual zoospores and maintained on straw in a defined liquid medium (14). Cultures have been maintained under conditions of cryopreservation by using techniques similar to those described previously (34).

Defined medium B and the aseptic, anaerobic techniques used in this work have been described previously (14, 18). Cultures were grown in thick-walled glass tubes (18 by 142 mm; Bellco Glass Inc., Vineland, N.J.) containing 150 mg of autoclaved, air-dried Italian ryegrass (*Lolium multiflorum* L.) hay (milled to pass through a 1-mm-diameter dry-mesh screen) and 15 ml of sterile medium. Cultures were incubated for up to 120 h under an atmosphere of 100% CO_2 at

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39°C without agitation. The entire time course experiment was repeated with a different *Neocallimastix* isolate. Although both sets of data were similar, only the set involving *Neocallimastix* sp. strain R1 is presented here.

Analysis of fermentation products and cell wall digestion. Cultures were harvested at 12-h intervals, and supernatants were separated from residual particulate substrate and adherent fungal biomass by centrifugation at 1,400 × *g* for 15 min. Supernatants from three replicate cultures were analyzed for the fermentation products formate and acetate as described previously (13, 17). To provide sufficient material for monosaccharide analysis, pellets from three to five replicate cultures were combined, washed, centrifuged twice more with 50-ml volumes of distilled water, and then lyophilized to constant weight. The lyophilized pellets (100 mg) were hydrolyzed under acidic conditions: 12 M H_2SO_4 for 1 h at 35°C followed by boiling in 1 M H_2SO_4 for 2 h. The resultant neutral sugars were derivatized to their alditol acetates and quantified as anhydro-sugars by gas chromatography (7). Each hydrolysate was derivatized and analyzed in duplicate; only the four major monosaccharides (arabinose, galactose, glucose, and xylose) in the Italian ryegrass hay cell walls were quantified. Since the cell walls of anaerobic fungi are composed predominantly of chitin (24) and because of the low biomass yields associated with anaerobic growth, it was assumed that sugars originating from fungal biomass and included in the lyophilized pellets made a negligible contribution to the total amounts measured.

First-order reaction kinetics and statistics. A Vax 11/750 computer (Digital Equipment Corp., Marlborough, Mass.) and a maximum likelihood program were used for fitting curves or lines to experimentally determined values. Data for the removal of cell wall sugars were analyzed by using first-order reaction kinetics which included a delay parameter to account for lag times (6). The extent of digestion was defined as the amount of neutral sugars removed 108 h after inoculation, since cell wall degradation approached an asymptote in 108 h or less and was considered to be complete in the culture system used. The difference between the amount of cell wall residue at 108 h and the initial amount represented the potentially digestible fraction. The rate constant for neutral-sugar loss was calculated as the slope of the line obtained by regressing the natural logarithm of the potentially digestible fraction against time. Fitted curves were compared by using parallel-curve analysis. This procedure required two steps. In the first step, fitted curves were made parallel by fixing the nonlinear parameter (the lag and rate parameter), generating curves of the same shape but not necessarily the same displacement. In the second step, the same curves were produced by fixing the nonlinear parameter and the linear parameter (the extent of digestion parameter), generating curves of the same shape and displacement, i.e., curves which were described by the same equations. The increase in residual mean squares associated with curves generated in these two steps was compared with the residual mean squares from individual (original) curves, and levels of significance were determined by using the F test.

Similar comparisons, involving parallel-curve analysis, were conducted on the regression lines which correlated formate accumulation with loss of cell wall dry weight and loss of neutral sugars from plant cell walls. In this case, the nonlinear parameter was represented by the slope of the regression line, whereas the linear parameter was represented by the intercept.

TABLE 1. Composition of Italian ryegrass hay

Component	Amt (% dry wt)
Soluble fraction	19.6
Insoluble fraction (plant cell walls)	
Glucose	35.0
Xylose	14.7
Arabinose	3.0
Galactose	0.8
Residue[a]	26.9

[a] The residue, which remained unaccounted for by monosaccharide analysis, was determined by difference and was presumed to consist mainly of lignin, minerals, protein, lipid, and other biopolymers.

RESULTS

Composition of undigested and digested cell walls. The insoluble fraction (cell walls) of Italian ryegrass hay, harvested from uninoculated culture tubes 24 h after autoclaving and aseptic addition of culture medium, represented 80.3% (wt/wt) of the original substrate (Table 1). Estimations of cellulose or hemicellulose contents were not attempted, because differential hydrolysis procedures were not used prior to the preparation of alditol acetate derivatives. However, the combined cellulose and hemicellulose fraction was quantified from the amounts of anhydro-monosaccharides (arabinose plus galactose plus glucose plus xylose) present in lyophilized cell wall hydrolysates. In the undigested substrate, the combined cellulose and hemicellulose fraction represented 53.5% (wt/wt) of the dry weight of Italian ryegrass hay and was composed of arabinose (5.6%, wt/wt), galactose (1.5%, wt/wt), glucose (65.4%, wt/wt), and xylose (27.5%, wt/wt) (Table 1). Trace amounts of rhamnose and mannose were detected during time course studies but were not quantified. A residual fraction, which remained unaccounted for by monosaccharide analysis and represented 26.9% (wt/wt) of the unfermented Italian ryegrass hay, was presumed to consist mainly of lignin, minerals, lipid, protein, and other biopolymers (Table 1).

Table 2 shows the composition of cell walls of Italian ryegrass hay harvested at 12-h intervals from 0 to 108 h after inoculation with *Neocallimastix* sp. strain R1. In comparison with undigested (zero time) cell walls, those harvested from the latter part of the fermentation period contained substantially smaller amounts of neutral sugars (cellulose and hemicellulose) and substantially more residual component (lignin, minerals, and other undigestible components). During the initial 60 h of the fermentation time course, however, when each of the individual sugars maintained an approximately constant proportion, there were relatively few differences between the neutral-sugar composition of undigested and digested cell walls (Table 2). Thereafter, the molar ratios showed a trend toward decreasing proportions of arabinose and glucose and increasing proportions of xylose with increasing fermentation time (Table 2).

Cell wall digestion. Previous studies have demonstrated that growth of *Neocallimastix* sp. strain R1 on plant cell walls results in a loss of cell wall dry weight and the production of fermentation end products (16, 17). Although formate, acetate, lactate, ethanol, carbon dioxide, and hydrogen all accumulate in batch cultures (31), only formate, which provides a reliable indicator of fungal biomass (16), and acetate were measured in the present study. Other volatile fatty acids, such as propionate and butyrate, were not detected in culture supernatants.

After an initial lag, the growth of *Neocallimastix* sp. strain R1 was accompanied by the accumulation of formate and

TABLE 2. Composition of plant cell walls of Italian ryegrass hay from cultures harvested at 12-h intervals from 0 to 108 h after inoculation with *Neocallimastix* sp. strain R1

Time since inoculation (h)	Amt of neutral sugars[a] (% dry wt)						Molar ratios	
	Arabinose	Galactose	Glucose	Xylose	Total[b]	Residue[c]	Glucose/xylose	Arabinose/xylose
0	3.8	1.0	43.6	18.3	66.7	33.3	1.98	0.21
12	3.5	1.0	43.6	18.4	66.5	33.5	1.97	0.19
24	3.6	1.0	45.5	18.5	68.6	31.4	2.05	0.19
36	2.7	1.0	44.4	16.7	64.8	35.2	2.26	0.16
48	2.8	0.9	39.4	15.5	58.6	41.4	2.12	0.18
60	2.4	0.8	33.0	14.4	50.6	49.4	1.93	0.17
72	1.8	0.7	26.6	12.0	41.1	58.9	1.85	0.15
84	1.3	0.6	24.7	12.6	39.2	60.8	1.65	0.11
96	1.7	0.6	25.9	13.0	41.2	58.8	1.66	0.13
108	1.1	0.6	22.1	13.0	36.8	63.2	1.41	0.08

[a] Each value is the mean of duplicate analyses.
[b] Sum of individual cell wall sugars. The standard deviation from the pooled differences over all times postinoculation was 2.82.
[c] Values unaccounted for by monosaccharide analysis and determined by difference (cell wall dry weight – total cell wall sugars).

acetate in culture media and the removal of dry weight and neutral sugars from the cell walls of Italian ryegrass hay (Fig. 1). To compare losses in dry weight and neutral sugars, results have been expressed as percentages of the corresponding amounts present in undigested (zero time) cell walls. Differences between the rate of removal of cell wall dry weight and the rate of removal of cell wall neutral sugars were attributed to the presence of an unfermentable fraction (residue), which formed an increasing proportion of the cell wall dry weight with increasing fermentation time (Fig. 1).

Results showing the loss of individual sugars (arabinose, galactose, glucose, and xylose) from plant cell walls are shown in Fig. 2. These data are presented on a percentage basis, as described above, and confirm that *Neocallimastix* sp. strain R1 was able to remove the four major cell wall monosaccharides of Italian ryegrass hay simultaneously and at similar rates. Curves (dashed lines in Fig. 2) were fitted to experimental values by using first-order reaction kinetics which included a delay parameter to account for lag times. In all four cases the fitted curves adequately described the experimental values for loss of individual sugars from plant cell walls. Values for the lag times, fractional disappearance rate constants, and extents of digestion for removal of sugars from plant cell walls were obtained from the fitted curves

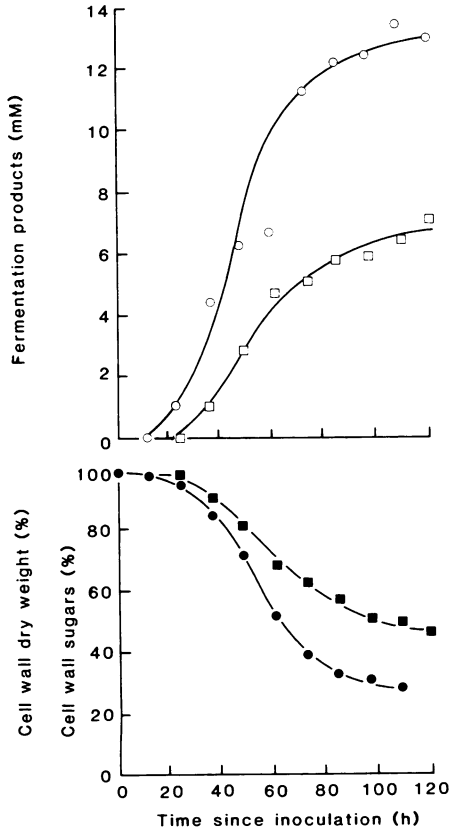

FIG. 1. Product accumulation and cell wall dry weight and cell wall sugar (arabinose plus galactose plus glucose plus xylose) losses during fermentation of Italian ryegrass hay by *Neocallimastix* sp. strain R1. Symbols: □, acetate; ○, formate; ■, residual cell walls; ●, residual sugars.

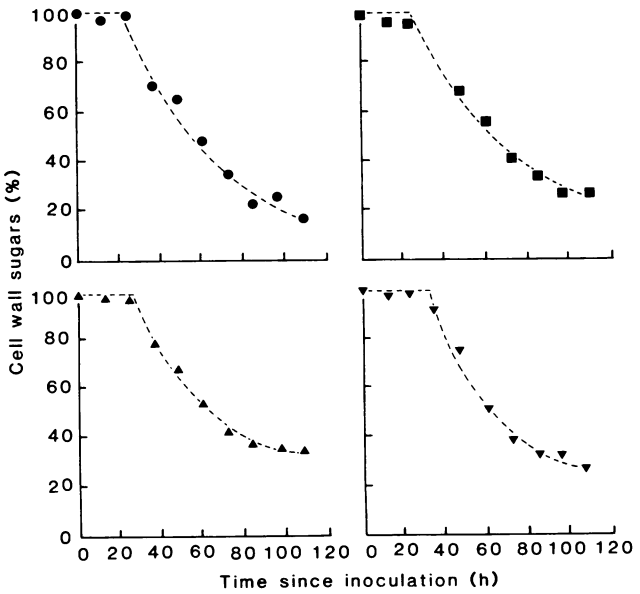

FIG. 2. Removal of individual cell wall sugars from Italian ryegrass hay during fermentation by *Neocallimastix* sp. strain R1. Symbols: ●, arabinose; ■, galactose; ▲, xylose; ▼, glucose; ----, curves fitted to experimental data according to a first-order reaction kinetics model which incorporated a lag.

TABLE 3. Digestion kinetics of Italian ryegrass hay cell walls

Sugar	Lag time (h)	Digestion rate constant (h^{-1})	Extent of digestion at 108 h (%)[a]
Arabinose	23.2	0.02	84.1(a)
Galactose	23.1	0.02	76.2(b)
Glucose	33.3	0.03	75.0(b)
Xylose	27.1	0.03	65.8(c)

[a] Values were obtained from curves fitted to experimental data by using a first-order reaction kinetics model which incorporated a lag. Data followed by different letters in the column were significantly different ($P < 0.05$) by parallel-curve analysis.

and are presented in Table 3. Although longer lag times and higher fractional disappearance rates were associated with the removal of glucose and xylose, they were not significantly different from those determined for the removal of arabinose and galactose (Table 3). Significant differences ($P > 0.05$) were obtained, however, between values for the extent of digestion of individual sugars from plant cell walls. Arabinose and xylose were the most and the least completely digested sugars, respectively, from the cell walls of Italian ryegrass hay (Table 3). Although experimental and predicted values for the removal of cell wall dry weight and cell wall sugars indicated that growth of *Neocallimastix* sp. strain R1 on Italian ryegrass hay was complete within 108 h in the culture system used, large amounts of potentially fermentable cell wall sugars remained at the end of the incubation period.

The relationships between formate accumulation, digested cell walls (milligrams [dry weight] lost from cell walls), and digested neutral sugars (milligrams of neutral sugars lost from cell walls) are presented in Fig. 3. Linear correlations ($r > 0.98$; $P < 0.001$) could be used to describe the relationship between formate accumulation and cell wall digestion and that between formate accumulation and neutral-sugar digestion. Even though values on the y axis in Fig. 3 were changed in progressing from one correlation to the other, the two relationships which described individual sets of data, i.e., formate accumulation = $[(0.42 \pm 0.619) + (0.24 \pm 0.017)] \times$ digested cell walls, and formate accumulation = $[(0.43 \pm 0.532) + (0.25 \pm 0.016)] \times$ digested neutral sugars, were not significantly different. Therefore, the regression line correlating the amount of digested cell walls with the amount of digested neutral sugars was not significantly different from 1.0, and the net effects of removal of dry weight from the cell walls of Italian ryegrass hay by *Neocallimastix* sp. strain R1 could be completely accounted for by the removal of four cell wall sugars.

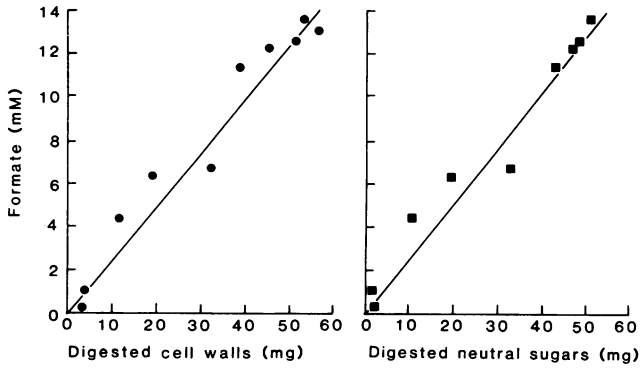

FIG. 3. Correlations between formate accumulation, digested cell wall dry weight, and digested cell wall sugars (arabinose plus galactose plus glucose plus xylose). Regression lines from each correlation were not significantly different when analyzed by parallel-curve analysis.

DISCUSSION

Neocallimastix sp. strain R1 was able to remove a considerable proportion of the arabinose, galactose, glucose, and xylose from the cell wall cellulose and hemicellulose of Italian ryegrass hay (Fig. 2 and 3; Table 3). These results provide the first account of the simultaneous removal of neutral sugars from plant cell walls by an anaerobic ruminal fungus. They are consistent with numerous reports in which mixed populations of ruminal bacteria have been shown to participate in the simultaneous removal of cell wall constituents (19, 20, 29, 32). Whether these components were removed as monosaccharide units or as oligosaccharides (or both) remains to be determined. However, rates of removal of the four neutral sugars were comparable to those usually associated with the removal of cell wall components from analogous forages by mixed populations of ruminal bacteria (4, 32). An observation also consistent with bacterial fermentations was the significantly reduced extent of digestion of xylose from plant cell walls when compared with that of the other three sugars (Table 3). It is well known that temperate forages consist of a heterogeneous population of cell types which, when analyzed after a period of microbial attack and compared with the original substrate, often show a trend toward increased xylose and decreased glucose content (9, 19, 29). This trend usually reflects the preferential degradation of primary cell walls, which, relative to secondary thickened and lignified walls, have a lower xylose content (9). Although factors other than the availability of cell wall sugars may contribute to limit the fermentation of plant cell walls in batch culture (31), it is unlikely that they would have made a significant contribution with the medium and substrate concentration used in the present study (M. K. Theodorou, unpublished results).

It has been shown previously that growth of *Neocallimastix* sp. strain R1 on wheat straw, wheat straw holocellulose, or cellulose resulted in substantial colonization of substrates and accumulation of fermentation products in culture media (16). The end product, formate, was correlated to a loss of substrate dry weight and could be used as a reliable indicator of fungal growth (16, 17). A similar correlation was evident in the present study, in which *Neocallimastix* sp. strain R1 removed ca. 53% (wt/wt) of the cell wall dry weight or ca. 75% (wt/wt) of the structural polysaccharides (cellulose and hemicellulose) of Italian ryegrass hay (Fig. 1; Table 3). Although the loss of cell wall dry weight must be viewed as a net effect (i.e., an apparent loss), representing cell wall losses as well as fungal biomass additions, the correlations presented in Fig. 3 suggest that cell wall components other than cellulose and hemicellulose were not removed to any significant extent by *Neocallimastix* sp. strain R1.

Anaerobic fungi and the major cellulolytic bacteria, *Ruminococcus albus*, *R. flavefaciens*, and *Bacteroides succinogenes*, produce a complex of fibrolytic enzymes necessary for the degradation of plant cell walls (5, 8, 16, 26–28). However, reports suggest that some cellulolytic bacteria are unable to ferment all of the sugars which they remove from plant cell walls (5, 27, 28). For example, *R. flavefaciens* can degrade hemicellulose and pectin, but not all of the liberated monosaccharides, including xylose, are fermented by this

bacterium (27, 28). Similarly, anaerobic fungi may be unable to utilize all of the reducing sugars which they liberate from plant cell walls. Although glucose and xylose are utilized by *Neocallimastix* sp. strain R1 as energy-yielding substrates, arabinose and galactose are not (17), and soluble sugars, including arabinose, have been shown to accumulate during wheat straw and xylan fermentations (16). An interaction between anaerobic fungi and methanogenic bacteria with respect to product formation and utilization has already been described (3, 21). In addition to demonstrating a potential method for cell wall degradation by anaerobic fungi in the rumen, the present research has highlighted the need to determine the form and the fate of the neutral sugars which are removed from plant cell walls.

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