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Cellulases and Xylanase of an Anaerobic Rumen Fungus Grown on Wheat Straw, Wheat Straw Holocellulose, Cellulose, and Xylan

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The activities of cellulolytic and xylanolytic enzymes produced by an anaerobic fungus (R1) which resembled *Neocallimastix* sp. were investigated. Carboxymethylcellulase (CMCase), cellobiase, and filter paper (FPase) activities had pH optima of 6.0, 5.5, and 6.0, respectively. CMCase and cellobiase activities both had a temperature optimum of 50°C, whereas FPase had an optimum of 45°C. The pH and temperature optima for xylanase activity were pH 6.0 and 50°C, respectively. Growth of the fungus on wheat straw, wheat straw holocellulose, or cellulose resulted in substantial colonization, with at least 43 to 58% losses in substrate dry matter and accumulation of comparable amounts of formate. This end product was correlated to apparent loss of substrate dry weight and could be used as an indicator of fungal growth. Milling of wheat straw did not enhance the rate or extent of substrate degradation. Growth of the R1 isolate on the above substrates or xylan also resulted in accumulation of high levels of xylanase activity and lower cellulase activities. Of the cellulases, CMCase was the most active and was associated with either low or trace amounts of cellobiase and FPase activities. During growth on xylan, reducing sugars, including arabinose and xylose, rapidly accumulated in the medium. Xylose and other reducing sugars, but not arabinose, were subsequently used for growth. Reducing sugars also accumulated, but not as rapidly, when the fungus was grown on wheat straw, wheat straw holocellulose, or cellulose. Xylanase activities detected during growth of R1 on media containing glucose, xylose, or cellobiose suggested that enzyme production was constitutive. Xylanase activity was mainly cell associated in these cultures, but there was a considerable increase in activity during fungal autolysis.

Several reports suggest that anaerobic fungi occupy a unique niche in the ruminal ecosystem and may function as the initial colonizers of lignocellulosic substrates (2–4). Anaerobic fungi can proliferate and survive in an ecosystem extensively colonized by fibrolytic bacteria and protozoa (1), and this may be related to their ability to penetrate and degrade plant tissues not normally accessible to other rumen microorganisms. Although research has indicated an important role for anaerobic fungi in the degradation and subsequent fermentation of particulate substrates in the rumen, comparatively few studies have been concerned with the production and activity of fibrolytic enzymes by these organisms (23, 27, 33). It has been shown that rumen fungi can grow in media containing cellulose or xylan (25), two important components of plant cell walls, and enhanced degradation of cellulose by *Neocallimastix frontalis* in the presence of methanogenic bacteria has been demonstrated (5). The extracellular cellulases of rumen fungi have also been characterized (23, 33), and carboxymethylcellulase (CMCase) activities have been demonstrated in supernatants from cultures grown on cellulose (23). The presence of glycosidases, including xylanase, have been reported for *N. frontalis* growing on cellulose (27).

In previous research (5, 23, 25, 27), cellulolytic and xylanolytic activities have been shown in vitro with filter paper and microcrystalline cellulose, substrates not normally encountered in the rumen. In this paper we describe the properties and production of cellulases and xylanase during growth of a rumen fungus (R1) on cellulose and xylan. We also describe growth on more natural carbon substrates such as wheat straw and wheat straw holocellulose. Since feed particles entering the rumen can be quite large, the effect of physical pretreatment of the substrate was also investigated by comparing the rate and extent of degradation of milled and unmilled wheat straw.

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MATERIALS AND METHODS

Medium, culture conditions, and culture maintenance. Defined medium B and the anaerobic culture techniques used in this work have been described previously (18). The *Neocallimastix* sp. described as R1 in the present study was isolated from the rumen of a sheep at The Animal and Grassland Research Institute (18; S. E. Lowe, G. G. Griffiths, A. Milne, M. K. Theodorou, and A. P. J. Trinci, J. Gen. Microbiol., in press).

Enzyme production and batch culture. Studies on pH and temperature optima and stability of extracellular enzymes were made with cell-free supernatants from cultures grown for 3 days in 100 ml of defined medium B in 125-ml serum bottles (Phase Separation Ltd.). Substrates for production of supernatants with cellulase and xylanase activities were Whatman no. 1 filter paper (0.5 g; 10 strips; ca. 6 by 1 cm) and 0.4% (wt/vol) larchwood xylan (Koch-Light), respectively. Culture supernatants were clarified by centrifugation (1,400 × *g*, 15 min). Media were inoculated with 10-ml zoospore suspensions obtained from cultures grown for 3 days in defined medium B containing 25 mM glucose; these cultures were in the late-exponential phase of growth and contained ca. 10^3 zoospores ml^{-1}.

Batch cultures for time course studies were grown on various carbon sources in anaerobic tubes (18 by 142 mm; Bellco Glass Inc.). For particulate substrates, tubes contain-

ing 0.1 g of substrate were autoclaved at 121°C for 15 min before addition of defined medium B (10 ml). All cultures were inoculated with a 1-ml zoospore suspension obtained as described above. The insoluble substrates were unmilled wheat straw (ca. 50 by 5 mm), hammer-milled wheat straw, wheat straw holocellulose (all generous gifts from D. M. Gaunt, University of Manchester), and crystalline cellulose (Avicel; Schleicher & Schuell Inc.). The wheat straw was prepared from *Triticum aestivum* cv. Avalon by stripping samples of leaf and ear remains; some of the substrate was hammer milled into approximately 0.5- to 1.0-mm^2 particles. Wheat straw holocellulose was prepared from hammer-milled wheat straw by removal of lignin as described by Lynch et al. (19). Cultures were also grown on xylan (0.4% wt/vol), cellobiose (13 mM), glucose (25 mM), or xylose (25 mM).

At intervals during time course studies, supernatants from batch cultures (three replicates) grown on particulate substrates were separated from residual substrate and fungal biomass by vacuum filtration through Whatman no. 1 filter paper disks (5.5-cm diameter). Pellets were washed with 50 ml of distilled water and dried to constant weight (80°C dry-heat oven). Supernatants were divided into three fractions; 1 ml was used immediately for xylanase assays, 2 ml was frozen at −20°C for subsequent formate and carbohydrate analyses, and 8 ml was frozen at −20°C for subsequent assays (up to 24 h later) of cellulase activities. The supernatants from cultures grown on xylan were separated from residual (undegraded) xylan by centrifugation at 1,400 × *g* for 15 min and divided into three fractions as described above. In these cultures, the fungal biomass which adhered to the sides of the glass tubes was harvested and dried to constant weight as described above.

Sonication. Fungal biomass from cultures grown on soluble sugars was harvested by centrifugation at 1,400 × *g* for 10 min, and the supernatants were assayed for various enzyme activities. Pellets from cultures grown on xylose were washed twice with 0.2 M citrate-phosphate buffer (pH 6.0) and suspended in 20 ml of the same buffer. Pellets from cultures grown on cellobiose were treated in the same way except that the buffer pH was 5.5. Pellets were sonicated with an ultrasonic disintegrator (model M284; MSE Scientific Instruments, Crawley, United Kingdom) (100 W; 6 × 30 s; 4°C). After centrifugation at 20,000 × *g* for 60 min, the sonicated supernatants were decanted and assayed for enzyme activities (soluble fraction). The residual pellets were washed twice in the appropriate buffer, suspended in an equal volume (20 ml) of buffer, and assayed for enzyme activities (insoluble fraction).

Chemical and biochemical assays. Culture supernatant was analyzed for reducing sugar with dinitrosalicylic acid (DNS) reagent (22); the ratio of sample to DNS reagent was 1:2, and absorbance was read at 640 nm. Glucose was determined with glucose oxidase-peroxidase with 2,2′-azino-di-(3-ethylbenzthiazoline)-6-sulfonate as the chromogen (7). Monosaccharides in culture supernatants were analyzed by gas-liquid chromatography with alditol acetate derivatives (11). Formate and protein were analyzed as described by Hopner and Knappe (14) and Bradford (8), respectively.

Enzyme assays. The filter paper assay (FPase) (20) was used to measure cellulase activity against crystalline cellulose. The assay consisted of Whatman no. 1 filter paper strips (6 by 1 cm; ca. 50 mg) coiled in the bottom of a test tube to which 1 ml of 0.2 M citrate-phosphate buffer (pH 6.0) was added. Culture supernatant (1 ml) was added to prewarmed substrate, and the reaction mixture was incubated at 45°C for 4 h. The reaction was terminated by addition of 4 ml of DNS reagent.

CMCase activity was measured with carboxymethylcellulose (30). The assay consisted of 10 mg of carboxymethylcellulose sodium salt (high viscosity; degree of substitution, >0.4; BDH) dissolved in 1.8 ml of 0.1 M citrate-phosphate buffer (pH 6.0). Culture supernatant (0.2 ml) was added to prewarmed substrate, and tubes were incubated at 50°C for 30 min. The reaction was terminated by addition of 4 ml of DNS reagent.

Cellobiase (β-glucosidase; EC 3.2.1.21) activity was determined by measuring glucose released from cellobiose (30). The reaction mixture consisted of 2 mg of cellobiose (BDH) in 1 ml of 0.2 M citrate-phosphate buffer (pH 5.5). Culture supernatant (1 ml) was added to pre-warmed substrate, and the reaction mixture was incubated at 50°C for 1 h. The reaction was terminated by placing tubes in a boiling water bath for 10 min. Glucose release was determined by the glucose oxidase-peroxidase method (7).

Xylanase activity was measured by incubating 0.2 ml of supernatant with 10 mg of xylan in 1.8 ml of 0.1 M citrate-phosphate buffer (pH 6.0) at 50°C for 30 min in a shaking water bath. The reaction was terminated by placing tubes in a boiling water bath for 10 min. Undegraded xylan was removed by centrifuging samples in a microcentrifuge (Micro Centaur; MSE Scientific Instruments) for 5 min, and the supernatant was analyzed for reducing sugar with DNS reagent.

All enzyme reactions were linear over the period of the assays. Cellulase and xylanase activities were expressed as micromoles of glucose (for cellulases) or xylose (for xylanase) released per min (international units) per ml of supernatant. Enzyme and substrate controls were included in all assays.

Anaerobic enzyme assays. Anaerobic assays were conducted by incubating reaction mixtures in anaerobic tubes under nitrogen; the buffer and substrates were also prepared under nitrogen. The resazurin (which was present in the culture medium as a redox indicator) remained colorless in assays conducted under nitrogen and was pink in assays conducted in air.

Cryo-SEM. Cultures grown on hammer-milled wheat straw were used for cryoscanning electron microscopy (cryo-SEM). Frozen, hydrated material was prepared in a manner similar to that described previously (32). Colonized particles of wheat straw were removed from culture tubes after 48 h of incubation, rinsed quickly in distilled water, and blotted dry on filter paper. These were attached to the specimen stub with carbon cement, frozen rapidly in nitrogen slush and, transferred under vacuum to the cooled prechamber of a Hexland CT1000 cryotransfer system which was interfaced to a Philips 505 scanning electron microscope. Excess water was sublimed away by heating to ca. −90°C, and the specimen was cooled to ca. −160°C and sputter coated with gold. Coated specimens were viewed by SEM at ca. −180°C.

Statistical analysis. A DEC VAX 11/750 computer and a maximum-likelihood program were used for statistical analysis. Data was fitted by using the logistics equation $Y = (A + C)/(1 + e^{-B(X - M)})$, where M = inflection point, B = shape parameter, and A and C are constants relating to the upper and lower asymptotes. Lack of fit was tested with the F test. A further test was conducted by parallel-curve analysis. Parallel curves were generated by fixing B and M (the nonlinear parameters). Thus, the same equation could describe all data sets. The increase in residual mean squares

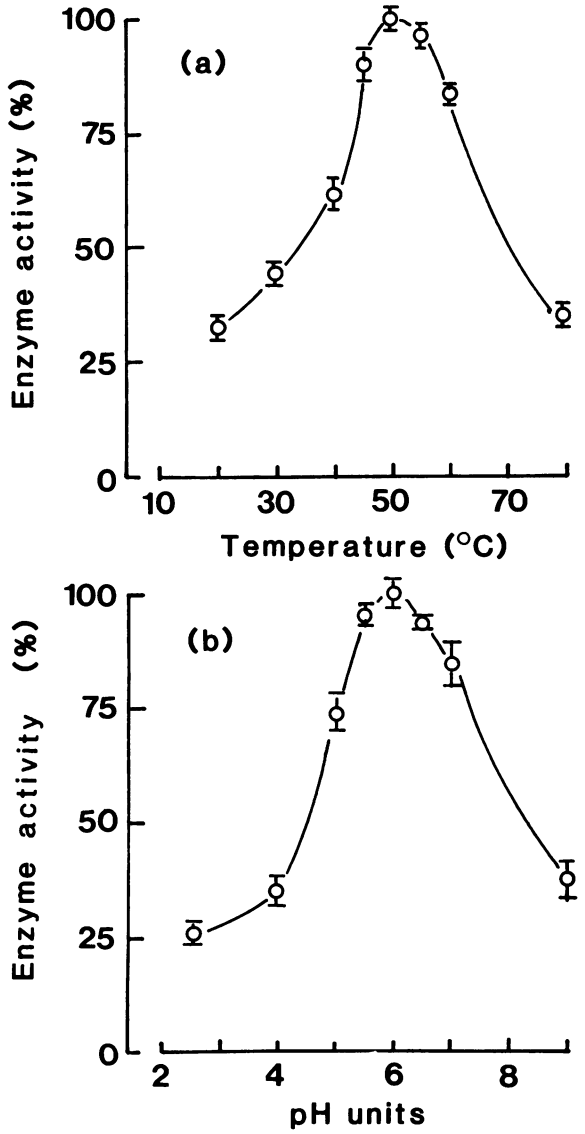

FIG. 1. Temperature (a) and pH (b) optima for xylanase activity of the R1 isolate. Xylanase activity was determined with supernatants from cultures grown for 3 days on xylan. Each value is the mean of three replicates. The vertical bars represent standard errors of the mean.

was compared with the residual mean squares associated with equations from individual curves by using the F test. Regression analysis was carried out to correlate the means of formate concentrations with the mean values for corresponding apparent loss of substrate dry weight.

RESULTS

The anaerobic fungus (R1) in the present work has been the subject of extensive studies (17; S. E. Lowe, Ph.D. thesis, University of Manchester, Manchester, England; Lowe et al., in press). The organism is similar to *N. frontalis* (13) but not *N. patriciarum* (26); however, there are sufficient morphological and biochemical differences to suggest that it is a new species.

Temperature and pH optima and enzyme stability. To determine the optimal temperature for enzyme activities, assays were performed at 20 to 80°C at pH 6.0, except for cellobiase assays, which were conducted at pH 5.5. For pH optima, assays were performed at 50°C, or at 45°C for FPase, over the pH range 2.5 to 9.0. Typical pH and temperature profiles for xylanase activity are presented in Fig. 1. Cellulase activities were determined with supernatants from cultures grown for 3 days on Whatman no. 1 filter paper. The pH and temperature optima of the cellulases were pH 6.0 and 45°C, pH 6.0 and 50°C, and pH 5.5 and 50°C for FPase, CMCase, and cellobiase, respectively.

Enzyme stability was investigated by incubating culture supernatants for up to 100 h at 50, 39, or 4°C in the absence of substrate. Relatively short incubation periods at 50°C resulted in substantial losses in enzyme activities, and at the end of a 7-h incubation period cellobiase, xylanase, and CMCase activities were reduced by 92, 58, and 30%, respectively. Similar losses in enzyme activities of 88, 68, and 18%, for cellobiase, xylanase, and CMCase respectively, occurred in supernatants incubated at 39°C, but longer incubation periods (up to 100 h) were required. Enzyme activities were more stable in supernatants incubated at 4°C, with negligible losses in cellobiase and CMCase activities and a 30% loss in xylanase activity after 100 h of incubation. Freezing (to −20°C) and thawing of culture supernatants resulted in ca. 21% loss in xylanase activity but had no appreciable effect on cellulase activities. The effect of oxygen on enzyme activities was investigated by assaying the culture superna-

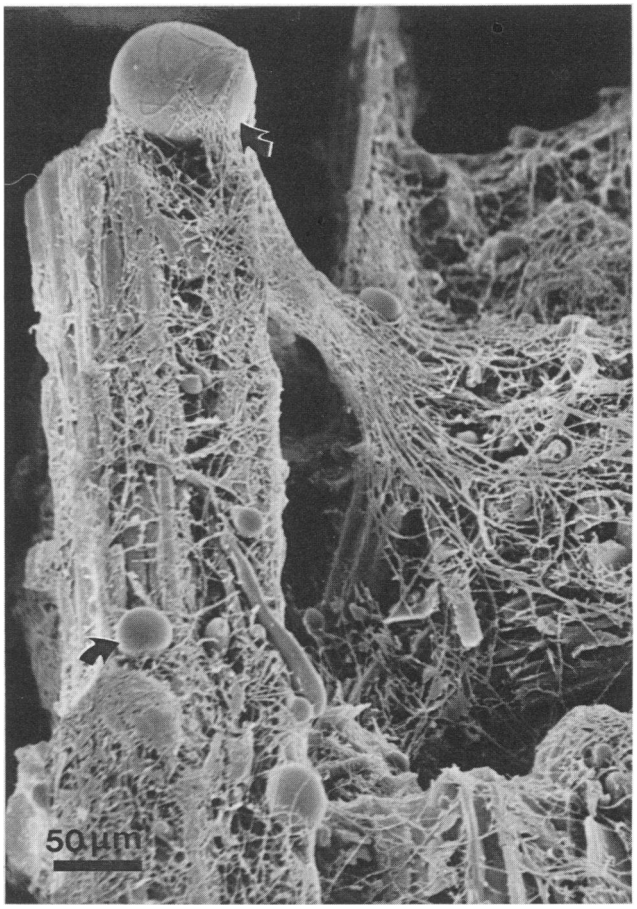

FIG. 2. Colonization of milled wheat straw after growth of R1 for 48 h as seen by cryo-SEM. Arrows indicate mature and immature zoosporangia which are associated with extensive rhizomycelium.

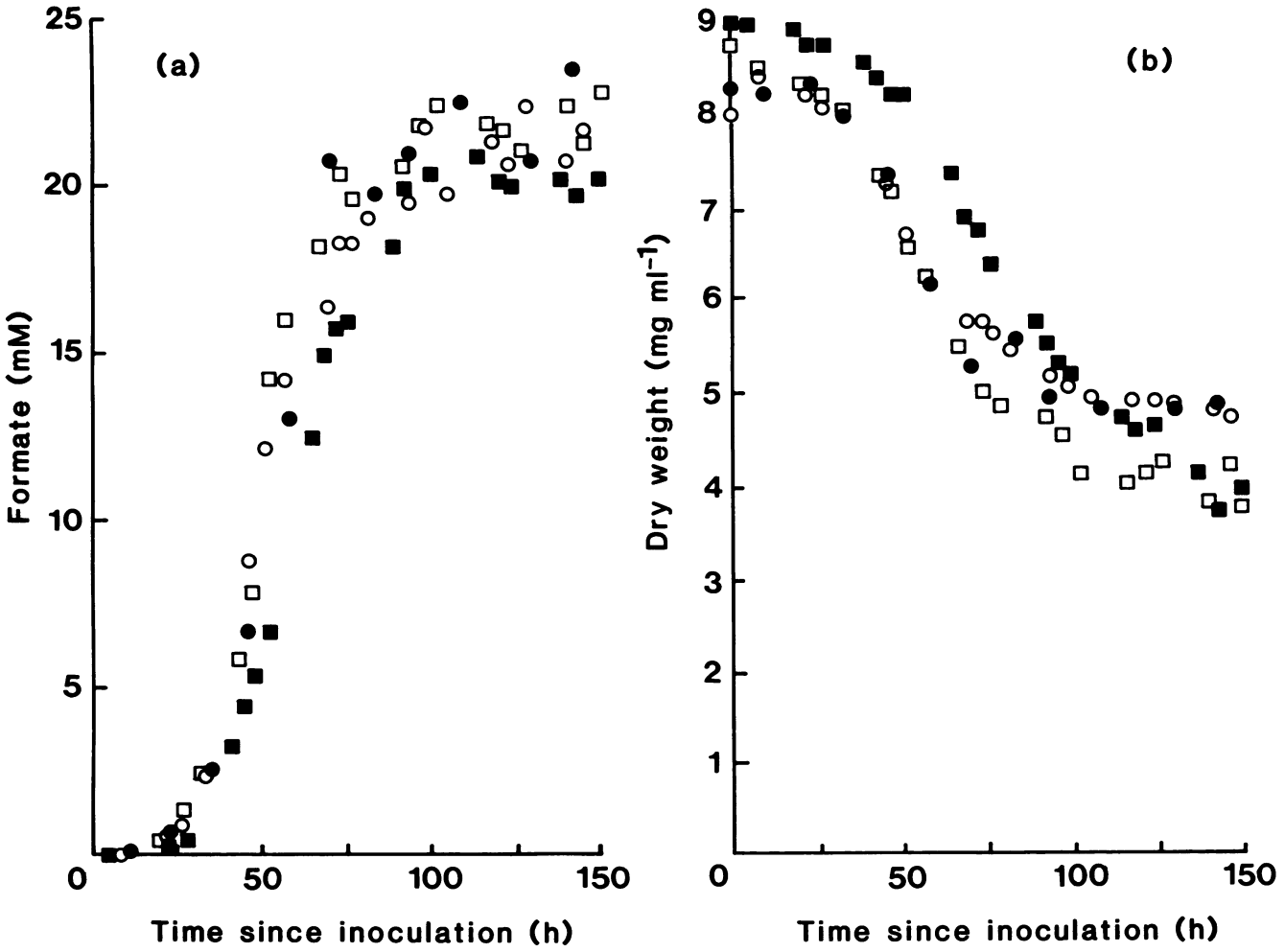

FIG. 3. Production of formate (a) and apparent loss of substrate dry weight (b) during growth of R1 on the following particulate substrates: unmilled wheat-straw, ●; milled wheat-straw, ○; wheat straw holocellulose, □; and cellulose, ■. Each value is the mean of three replicates.

tants under aerobic and anaerobic conditions, and significant differences were not observed. All enzyme assays were subsequently made aerobically under optimal temperature and pH conditions; supernatants for xylanase activity were assayed immediately, whereas supernatants for CMCase, FPase, and cellobiase activities were frozen (at −20°C) for up to 24 h before assay.

Growth on insoluble substrates. The extent of colonization of milled wheat straw was demonstrated qualitatively after 48 h of incubation by cyro-SEM (Fig. 2). Straw particles were intimately associated with an extensive network of colonizing rhizoids supporting numerous mature and immature zoosporangia. Because the fungus was inseparable from undegraded substrate, biomass production and substrate utilization could not be quantified by direct measurement. Previous studies have shown that fungal biomass was directly proportional to formate accumulation during active growth of R1 on glucose (17). Therefore, in the present study, formate accumulation and apparent loss of substrate dry weight were used as indirect indicators of fungal growth and substrate utilization, respectively.

In cultures containing unmilled wheat straw, milled wheat straw, wheat straw holocellulose, or cellulose, formate accumulation began ca. 25 h after inoculation and continued for ca. 80 h thereafter, reaching maximal values of ca. 22 mM (Fig. 3a). Substrate utilization coincided with formate accumulation, and substrate dry weights were reduced by at least 43 to 58% by the end of the fermentations (Fig. 3b). Regression analysis of dry weight (milligrams per milliliter) versus formate (millimolar) showed that apparent loss of substrate dry weight was proportional to formate accumulation during active growth of the R1 isolate on particulate substrates. Furthermore, a common expression, $F = (-5.7 \pm 0.23)S + (50.8 \pm 1.61)$, where F = formate concentration (millimolar) and S = apparent dry weight of substrate (milligrams per milliliter), could be used to describe the first 70 h of growth of R1 on all substrates. However, apparent dry weight loss and formate accumulation were not proportional during the latter part of the fermentation, and it was notable with certain substrates (particularly cellulose) that apparent dry weight loss continued in the absence of formate accumulation.

Data for formate accumulation and apparent loss of substrate dry weight were fitted to curves by using the logistics equation and subjected to analysis of variance. In each case, fitted values were not significantly different from their corresponding experimental values. Curves were also analyzed by parallel-curve analysis. Curves generated from data obtained by fermentation of milled and unmilled wheat straw were not significantly different; i.e., the rates and extents of

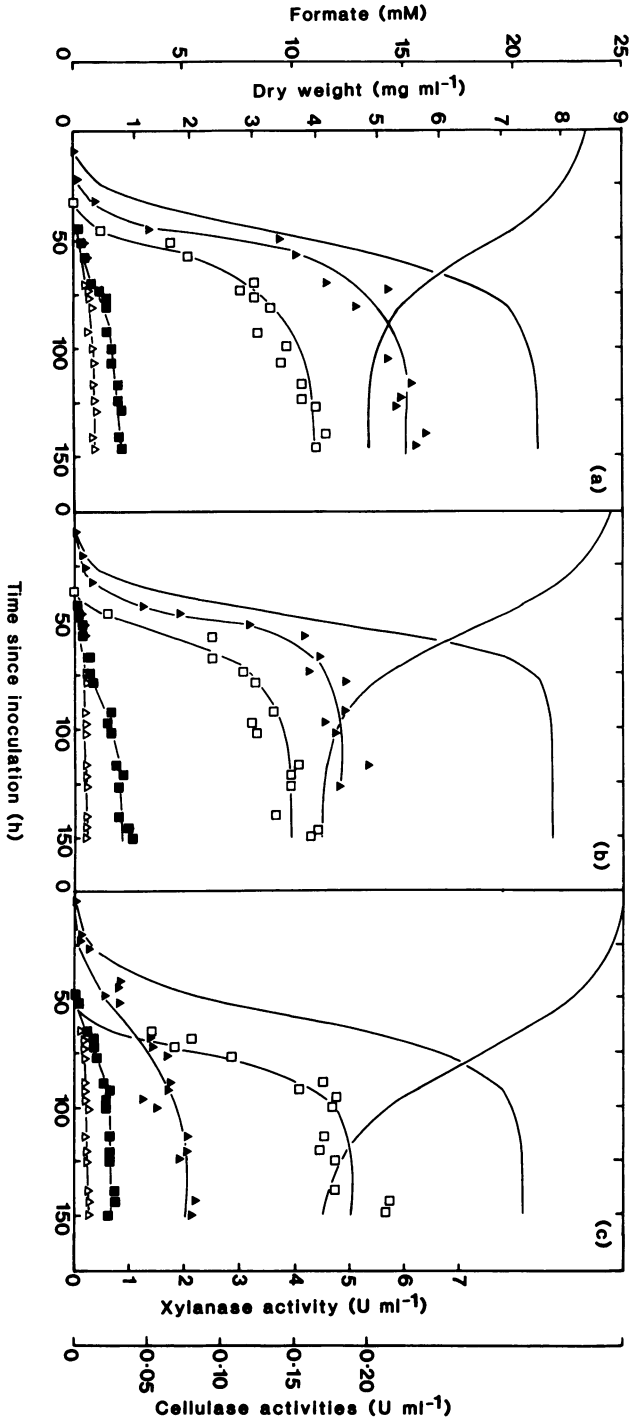

FIG. 4. Growth and production of extracellular cellulases and xylanase by R1 on the following particulate substrates: milled wheat-straw (a), wheat straw holocellulose (b), and cellulose (c). Fitted curves for formate production and apparent dry weight loss for each substrate were obtained from the data in Fig. 3. Enzyme activities were determined from culture supernatants and include xylanase (▲), CMCase (□), cellobiase (■), and FPase (△). Each value is the mean of three replicates.

formate accumulation and apparent loss of substrate dry weight in these two fermentations were the same. Significant differences ($P < 0.05$) were obtained, however, when comparing curves from wheat straw fermentations with those from the fermentation of cellulose or wheat straw holocellulose. In comparison with wheat straw fermentations, the rates and extents of formate accumulation and apparent dry weight loss were significantly lower ($P < 0.05$) on cellulose and significantly higher on wheat straw holocellulose.

Enzyme activities on insoluble substrates. For comparisons of formate accumulation, apparent loss of substrate dry weight, and enzyme activities during growth on milled wheat straw, wheat straw holocellulose, and cellulose, fitted curves from the data in Fig. 3a and b are presented in Fig. 4a to c, together with data from enzyme assays. Because of the similarities between milled and unmilled wheat straw fermentations, enzyme assays were not performed on supernatants from the latter substrate. In all fermentations, changes in enzyme activities and formate concentrations in culture supernatants and apparent dry weight loss in culture solids were generally related. Thus, as formate accumulated, enzyme activities increased and the dry weight of culture solids decreased (Fig. 4a to c). Xylanase activity began to accumulate ca. 20 h after inoculation, but cellulase activities were generally not detected until ca. 20 h thereafter. Fermentation of milled wheat straw and wheat straw holocellulose resulted in accumulation of 6.0 and 4.9 IU of xylanase activity per ml, respectively; these were higher than the maximum xylanase activity (2.0 IU ml^{-1}) achieved during fermentation of cellulose (Fig. 4c). Fermentation of each substrate resulted in accumulation of similar cellulase activities, which were considerably lower than corresponding values for xylanase activity (Fig. 4a to c). CMCase was the most active of the cellulases, achieving maximum activities of 0.16, 0.14, and 0.19 IU ml^{-1} during fermentations of milled wheat straw, wheat straw holocellulose,and cellulose, respectively. During these fermentations, approximately 0.03 IU of cellobiase activity per ml and 0.01 IU of FPase activity per ml accumulated (Fig. 4a to c).

Culture supernatants were also screened for reducing sugars, which began to accumulate during growth on milled wheat straw or wheat straw holocellulose ca. 40 h after inoculation. By the end of the fermentations, reducing sugar concentrations were 0.28 and 0.75 mg ml^{-1}, respectively (Fig. 5a and b). Reducing sugars were also detected during cellulose fermentations, although not until 75 h after inoculation. By the end of cellulose fermentation, 1.38 mg of reducing sugar per ml had accumulated (Fig. 5c), 70% of which was detected as glucose.

Growth and enzyme activity on xylan. Growth and enzyme activity on xylan and accumulation of reducing sugars are shown in Fig. 6. The increase in fungal dry weight (Fig. 6a) could be determined because the R1 isolate grew on the glass walls of anaerobic tubes and could be separated from residual xylan in culture supernatants. Cultures had a doubling time of 8.01 h and achieved a maximum biomass concentration of 0.57 mg ml^{-1} after 66 h of incubation. A loss in dry weight toward the end of fermentation indicated fungal autolysis (Fig. 6a). Extracellular xylanase activity was detected in supernatants 22 h after inoculation and increased during the growth and autolytic phases, reaching a maximum activity of 3.2 IU ml^{-1}. CMCase activity was detected 48 h after inoculation, and the maximum activity (0.1 IU ml^{-1}) was detected ca. 18 h later (Fig. 6a). Detection of trace

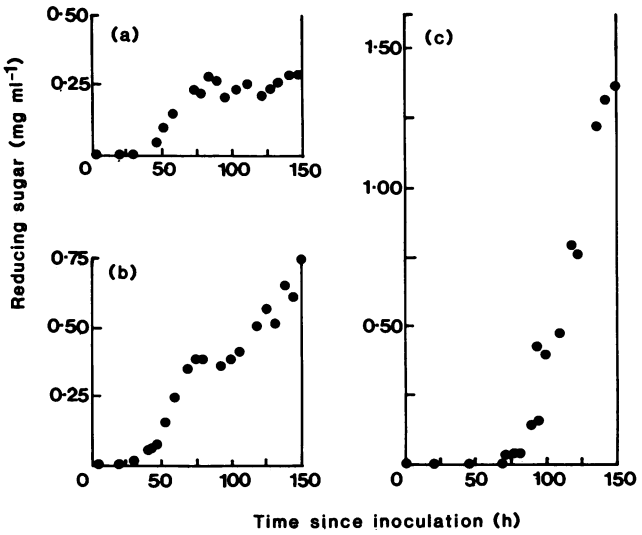

FIG. 5. Accumulation of reducing sugars during growth of K1 on particulate substrates. Values for milled wheat straw (a), wheat straw holocellulose (b), and cellulose (c) were determined from fermentations described in Fig. 3. Each value is the mean of three replicates.

amounts of cellobiase and FPase activity in supernatants coincided with the appearance of CMCase activity (Fig. 6a).

Growth on xylan was also accompanied by accumulation of xylose, arabinose, and other reducing sugars (Fig. 6b). Xylose concentrations increased and then declined, but arabinose continued to accumulate throughout active growth and did not decline during autolysis (Fig. 6b). Not all of the reducing sugar could be accounted for by xylose and arabinose alone, but the pattern of reducing sugar accumulation was similar to the sum of the individual monosaccharides.

Growth and enzyme activity on glucose, xylose, or cellobiose. To investigate the possibility that xylanase was constitutive, R1 was grown on glucose, xylose, or cellobiose, and culture supernatants were assayed for xylanase activities. Cellobiase activity was determined during growth of R1 on cellobiose.

Significant xylanase activities were detected when the fungus was grown on all three sugars, but the highest xylanase activity (2.29 IU ml^{-1}) was observed when the organism was grown on xylose (xylanase activities on glucose and cellobiose were 1.19 and 1.32 IU ml^{-1}, respectively). The greatest increase in xylanase activity was observed during the autolytic phase of growth. Only trace amounts of cellobiase activity (0.008 IU ml^{-1}) were detected in the culture supernatant during growth of R1 on cellobiose, and there was no increase in activity during the autolytic phase of growth.

Increased xylanase activity during autolysis, as well as low levels of cellobiase activity during growth on cellobiose (and on cellulose), suggested that these enzymes may be cell associated. To investigate this, the fungus was grown on xylose or cellobiose for 54 h (to ensure that the culture was in the mid-exponential and not the autolytic phase of growth), and culture supernatants were assayed for xylanase and cellobiase activities, respectively, before and after sonication (Table 1). Microscopic examination of the insoluble fraction after sonication showed fragmented hyphae and zoosporangia (which were absent in the soluble fraction). Protein concentrations were determined after each sonication cycle, and values became constant after five cycles. Cellobiase activity was not detected in supernatants of cultures incubated for 54 h on cellobiose before sonication, although low levels of xylanase activity (0.28 IU ml^{-1}) were detected in the supernatant from cultures grown on xylose. After sonication, the cellobiase activity of the supernatant was 0.016 U ml^{-1}, whereas the activity of the insoluble fraction was 0.115 U ml^{-1}. Xylanase activities of

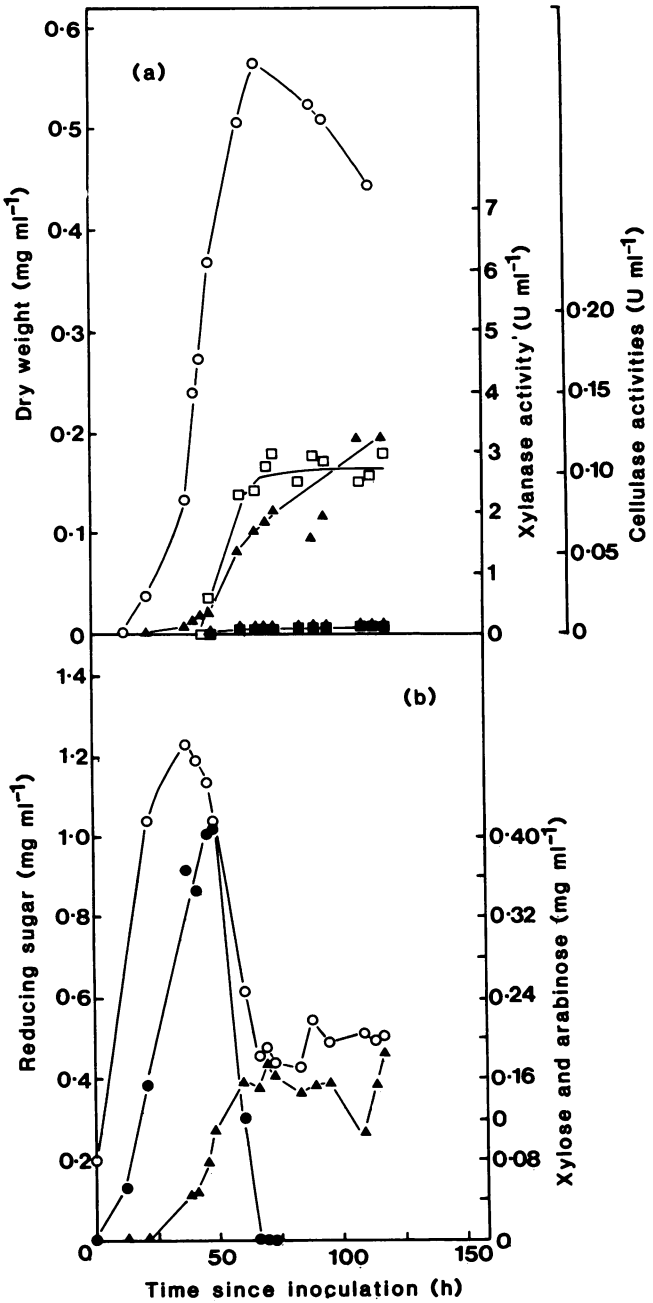

FIG. 6. Growth, enzyme activities, and accumulation of reducing sugars during growth of the R1 isolate on xylan. (a) Symbols: dry weight, ○; xylanase, ▲; CMCase, □; cellobiase, ■; and FPase, △. (b) Reducing sugar, (○), xylose (●), and arabinose (▲) were determined from the culture supernatant of each tube. Each value is the mean of three replicates.

TABLE 1. Location of cellulases and xylanase[a]

Enzyme	Mean (± SEM) enzyme activity (IU ml^{-1})		
	Extracellular enzymes[b]	Soluble fraction[c]	Insoluble fraction[d]
Cellobiase	0	0.016 ± 0.0002	0.115 ± 0.0050
Xylanase	0.280 ± 0.016	0.507 ± 0.011	1.007 ± 0.040

[a] Cultures were grown for 54 h on 13 mM cellobiose or 25 mM xylose and assayed for cellobiase and xylanase activities, respectively.
[b] Supernatant of the culture medium assayed for extracellular enzymes.
[c] The cells were sonicated and centrifuged, and the supernatant was assayed (soluble fraction).
[d] The pellet was suspended in an equal volume of buffer and assayed for enzyme activity (insoluble fraction). Each value is the mean of 10 replicates.

0.507 and 1.007 U ml^{-1} were recorded in the soluble and insoluble fractions, respectively.

DISCUSSION

The pH and temperature optima of cellulases from the R1 isolate and the effect of temperature on enzyme stability were in general agreement with results for *N. frontalis* (PN-1 and PN-2) (23). However, the FPase activity of R1 had pH and temperature optima of pH 6.0 and 45°C respectively, which differed from those for Avicelase activity (pH 5.5 and 50°C) reported for *N. frontalis* (23). The optimum pH and temperature for xylanase activity of R1 (pH 6.0 and 50°C) also differed from those reported (pH 5.1 and 37°C) for *N. frontalis* PN-2 (27). Cellulases (in particular cellobiase) and xylanase were unstable in the absence of substrate at 39 and 50°C; similar results have been reported for the CMCase of *N. frontalis* (23). Incubation of cellulases and xylanase under aerobic as opposed to anaerobic conditions had no significant effect on enzyme activities. This has also been reported for CMCase from *N. frontalis* (23).

During unmilled and milled wheat straw fermentations, values for apparent loss of substrate dry weight or formate accumulation were not significantly different. Furthermore, the R1 isolate was able to utilize all four particulate substrates in a similar fashion, and a single relationship could be used to correlate apparent loss of substrate dry weight and formate accumulation during the initial 70 h of each fermentation. These results clearly demonstrate the ability of anaerobic fungi such as R1 to degrade complex substrates. In a similar study with identical substrates, *Trichoderma reesei* degraded at least 15 and 75% of milled wheat straw and wheat straw holocellulose, respectively (D. M. Gaunt, Ph.D. thesis, University of Manchester, 1986). Comparison of these values with those obtained for R1 showed that the rumen fungus can utilize considerably more wheat straw than can *T. reesei* but less wheat straw holocellulose. Chlorite-treated wheat straw (wheat straw holocellulose) was used as an indirect way of investigating the inhibitory effects of aromatic complexes on straw digestibility by rumen fungi. It should be noted, however, that although chlorite pretreatment does maximize the amount of carbohydrate in the resultant holocellulose by oxidation of certain aromatic structures, significant structural changes in cell wall polysaccharides also occur (12).

There have been reports that lignin has an inhibitory effect on enzyme production (21). Other workers (31) found that levels of extracellular cellulases and xylanases produced by *Aspergillus fumigatus* decreased with removal of lignin from straw and hay. Although the levels of extracellular enzymes produced when the R1 isolate was grown on milled wheat straw were higher than for cultures grown on wheat straw holocellulose, the latter substrate was degraded to a greater extent. This difference in dry weight loss could be accounted for by the residual, undegraded lignin component in milled wheat straw.

Several explanations have been suggested to account for the anomaly of xylanase production when organisms are grown on cellulose. Lynch et al. (19) reported that xylose was a contaminant in Whatman no. 1 filter paper and may act as an inducer of xylanase. However, the use of highly purified microcrystalline cellulose (Avicel) in the present study, together with the production of xylanase on media containing glucose or cellobiose, suggests that the xylanase of R1 is constitutive. Constitutive xylanase production has been reported for a number of other organisms (6, 16). Alternatively, it is possible that some cellulases act in a nonspecific manner and cleave the β,1-4 linkage of the xylan molecule (10). Two xylanases which attack xylan and crystalline cellulose have been found in *A. niger* (15). A further explanation for the production of xylanase with cellulases in R1 is that the enzymes may be produced and regulated in a coordinate manner, as in *T. viride* (24).

Growth of the R1 isolate resulted in accumulation of reducing sugars in culture supernatants (Fig. 5). Similar results have been reported for *N. frontalis* growing on filter paper (23, 27), although unlike results in the present study, reducing sugars were not detected during growth on Avicel (27). Differences in the time of appearance and pattern of reducing sugar accumulation during growth of R1 suggest that initial degradation of xylan was rapid, whereas milled wheat straw, wheat straw holocellulose, and cellulose were degraded more slowly. Although xylose and some reducing sugars were subsequently utilized by R1 during xylan fermentations, arabinose continued to accumulate (Fig. 6). This was consistent with previous results (17) in which the R1 isolate failed to grow on arabinose (and some other monosaccharides). Liberation of nonutilizable mono- and oligosaccharides could account for the accumulation of reducing sugars during milled wheat straw and wheat straw holocellulose fermentations. However, since the R1 isolate can utilize glucose and cellobiose (17), the above explanation cannot account for reducing sugar accumulation (predominantly glucose) during the latter part of cellulose fermentation. Thus, it appears likely that growth on cellulose was limited by factors other than the availability of carbon. Since CMCase activity has been shown to be unaffected in the presence of high levels (1 mg ml^{-1}) of glucose (Lowe, Ph.D. thesis), end product inhibition of enzymes could not account for the cessation of growth of R1 on cellulose. However, high levels of fermentation products (such as formate) could inhibit fungal growth since it has been shown that coculture of *N. frontalis* with formate- and hydrogen-utilizing methanogens significantly enhanced cellulose utilization (5).

The increased levels of xylanase activity in culture supernatant during autolysis, together with the increase in xylanase activity after sonication, suggest that xylanase is partly extracellular and partly cell associated. There are a number of reports of rumen bacteria producing cell-associated xylanases (9, 28, 29).

Growth of R1 on unmilled wheat straw demonstrates the ability of rumen fungi to colonize and degrade plant material. The high levels of xylanase produced and the constitutive nature of this enzyme suggest a significant role for the R1 isolate in xylan degradation. These findings support the suggestion that anaerobic fungi are important in ligno-

cellulose degradation in the rumen and may also participate in the utilization of hemicellulose.

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