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Microbial Delignification with White Rot Fungi Improves Forage Digestibility

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Three wild-type white rot fungi and two cellulase-less mutants developed from *Phanerochaete chrysosporium* K-3 (formerly *Sporotrichum pulverulentum*) were tested for their ability to delignify grass cell walls and improve biodegradation by rumen microorganisms. Fungal-treated and control stems of Bermuda grass were analyzed for their content of ester- and ether-linked aromatics by using alkali extraction and gas chromatography, for in vitro dry weight digestion and production of volatile fatty acids in in vitro fermentations with mixed ruminal microorganisms, for loss of lignin and other aromatics from specific cell wall types by using microspectrophotometry, and for structural changes before and after in vitro degradation by rumen microorganisms by using transmission electron microscopy. *P. chrysosporium* K-3 and *Ceriporiopsis subvermispora* FP 90031-sp produced the greatest losses in lignin and improved the biodegradation of Bermuda grass over that of untreated control substrate. However, *C. subvermispora* removed the most lignin and significantly improved biodegradation over all other treatments. *Phellinus pini* RAB-83-19 and cellulase-less mutants 3113 and 85118 developed from *P. chrysosporium* K-3 did not improve the biodegradation of Bermuda grass lignocellulose. Results indicated that *C. subvermispora* extensively removed ester-linked *p*-coumaric and ferulic acids and also removed the greatest amount of non-ester-linked aromatics from plant cell walls. Microscopic observations further indicated that *C. subvermispora* removed esters from parenchyma cell walls as well as esters and lignin from the more recalcitrant cell walls (i.e., sclerenchyma and vascular tissues). *C. subvermispora* improved in vitro digestion and volatile fatty acid production by ruminal microorganisms by about 80%, while dry matter loss due to fungi was about 20% greater than loss in untreated control stems. The chemical and structural studies used identified sites of specific fungal attack and suggested mechanisms whereby improvement occurred.

It is well established that white rot fungi are capable of degrading lignin in plant cell walls (11, 18, 21, 22, 39). The common pattern of attack on lignocellulose by white rot fungi is a simultaneous decay of polysaccharides and lignin, but preferential degradation of lignin may also occur (22). Patterns of decay and degrees of delignification vary, however, for different fungal species and even strains of a species (13). Some species selectively delignify plant fiber, thus exposing an unprotected and available carbohydrate (13). Therefore, it is necessary to test specific fungi and substrates to find those best suited to the purpose of biological delignification.

Much of the reported research has dealt with delignification of wood by white rot fungi, particularly *Phanerochaete chrysosporium* (21). However, some research has been undertaken to biologically delignify herbaceous plants to improve the utilization of lignocellulose by ruminants. Zadrazil (43) evaluated 235 strains of fungi for their ability to delignify wheat straw and reported extreme variations in activity, with substantial influences by temperature. Jung et al. (33) reported both increased and decreased in vitro digestibility of oat straw after pretreatment with different fungi, with the improved digestibility being offset by a substantial loss of dry matter in the fungal treatment. Further work by Jung and coworkers (34), using *P. chrysosporium* to biologically delignify grass and legume cell walls, resulted in loss of more polysaccharides than lignin and did not improve digestibility by rumen microorganisms under their conditions. Agosin et al. (1) found that nonselective degradation of plants (i.e., loss of hemicelluloses and cellulose along with lignin) by *P. chrysosporium* (previously called *Sporotrichum pulverulentum*) resulted in small increases in in vitro digestibility by ruminal microorganisms. In contrast, two other white rot fungi selectively degraded hemicellulosic sugars and aromatic constituents, caused low dry-weight losses, and improved in vitro digestibility by 63 and 94% (1). Similarly, Karunanandaa et al. (35) reported increases and decreases in in vitro ruminal digestibilities of corn and rice straw after pretreatment with various fungi; where improved digestibility occurred, the fungi selectively used the hemicelluloses rather than cellulose.

Bermuda grass is a warm-season grass used extensively as a ruminant feed in tropical and subtropical regions (10). Considerable research has been done on the sites and types of lignin within cell walls (6, 25). The objective of our research was to test a series of white rot fungi, including *P. chrysosporium* and two cellulase-less mutants, for their ability to remove lignin and other aromatics from stem cell walls and to improve the utilization of the lignocellulose residues by mixed ruminal microorganisms.

MATERIALS AND METHODS

Plant substrate. Coastal Bermuda grass (*Cynodon dactylon* L. Pers) was harvested from well-managed experimental plots in Athens, Georgia, at 6-week regrowth. Shoots were stored at −10°C until used. The lower stem internodes

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TABLE 1. Amounts of aromatic compounds in Bermuda grass stems after fungal treatment, determined by alkali extraction

Fungal treatment	Amount of indicated compound[a] determined by indicated extraction treatment								
	Sequential[b]								Direct[c]
	1 M NaOH				4 M NaOH				
	Ester		Soluble lignin[d]		PCA	FA	G	S	
	PCA	FA	G	S					
None (untreated control)	9.4 ± 0.1 a	3.6 ± 0 a	0.2	1.3	2.4 ± 0 a	2.0 ± 0.1 a	2.1 ± 0.1 a,b	3.1 ± 0.1 a,b	22.9 ± 2.5 a,b
P. chrysosporium K-3	6.1 ± 0.2 b	1.0 ± 0.2 b	Trace	2.7	1.7 ± 0.3 a	1.5 ± 0.2 a	2.1 ± 0.3 a,b	2.4 ± 0 b	18.9 ± 1.0 c,d
P. chrysosporium K-3 mutant 3113	9.9 ± 1.0 a	2.0 ± 0.8 c	0.8	2.5	2.8 ± 0.1 a	2.7 ± 0.2 b	1.6 ± 0.6 b	2.6 ± 1.0 b	25.0 ± 1.2 a
P. chrysosporium K-3 mutant 85118	6.9 ± 0.6 b	2.1 ± 0.2 c	Trace	1.1	2.5 ± 1.0 a	2.1 ± 0.6 a,b	2.8 ± 0.3 a	4.0 ± 0.9 a	21.3 ± 1.1 b,c
P. pini RAB-83-19	7.5 ± 0.7 b	2.0 ± 0.3 c	0.1	1.6	2.2 ± 0.1 a	2.0 ± 0.1 a	2.0 ± 0.1 b	3.1 ± 0.2 a,b	22.3 ± 0.6 a,b
C. subvermispora FP 90031-sp	4.5 ± 0.2 c	0.8 ± 0 b	0	3.1	1.8 ± 0.3 a	1.6 ± 0.2 a	1.6 ± 0.2 b	2.7 ± 0.2 a,b	17.8 ± 0.9 d

[a] In milligrams per gram (average ± standard deviation of duplicate tubes). Values within a column with different postscript letters (a, b, c, and d) differ at $P \leq 0.05$.
[b] PCA, *p*-coumaric acid; FA, ferulic acid; G, guaiacyl units; S, syringyl units.
[c] Treatment with 4 M NaOH directly.
[d] Soluble lignin = amount from direct 4 M treatment − amount from sequential 4 M treatment, for G and S.

(internodes 4 and 5 from the apex) were selected, and 10-mm-long sections were excised from these internodes for pretreatment and further study. This position on the stem was chosen to have samples of high lignin content and low biodegradability. Comparable samples of similar stem material showed that cell walls had about 25% (wt/wt) lignin as determined by the $KMnO_4$ method (9). The sections were sterilized with ethylene oxide in a commercial cannister (Ben Venue Laboratories, Inc., Bedford, Ohio; model no. VIII) and then aseptically placed on agar plates (2% wt/vol) (Difco, Detroit, Mich.) with no addition of nutrients. For each treatment, 125 sections for each of 4 plates were prepared.

Fungal treatment. The white rot fungi used to pretreat Bermuda grass stems were as follows: (i) *P. chrysosporium* K-3, (ii) cellulase-less mutant 3113 from *P. chrysosporium* K-3 (31), (iii) cellulase-less mutant 85118-b from *P. chrysosporium* K-3 (31) (mutants 3113 and 85118-b were both obtained by courtesy of C. Johnsrud, Swedish Pulp and Paper Institute, Stockholm), (iv) *Phellinus pini* RAB-83-19, and (v) *Ceriporiopsis subvermispora* FP 90031-sp (*P. pini* and *C. subvermispora* were both obtained by courtesy of R. A. Blanchette, University of Minnesota). The five organisms are referred to below, respectively, as K-3, 3113, 85118-b, RAB-83-19, and FP 90031-sp. Control samples of Bermuda grass without fungal inoculation were maintained and treated as the others.

For inoculation, small squares of agar blocks (malt extract agar; Difco) with the fungus were placed onto agar plates with the sterilized stem sections, which covered the agar surface. Plates were enclosed in plastic bags and incubated at 39°C (K-3, 3113, and 85118-b) or 27°C (RAB-83-19 and FP 90031-sp). Plates were incubated for 6 weeks, after which time sections were retrieved. A portion of the sections was frozen, and the other portion was freeze-dried and ground (1-mm mesh) for analyses.

Alkali-labile aromatics in Bermuda grass stem residues. Ground stems were analyzed for lignin and other aromatics remaining in the fungus-treated and untreated residues according to published procedures (30). Briefly, aromatic constituents linked by ester bonds were assessed by treatment of residues with 1 M NaOH at 25°C for 24 h. This alkali-treated material was then sequentially treated with 4 M NaOH at 170°C for 2 h in a Teflon vial contained in a stainless steel reaction vessel to assess ether-linked aromatics. Additionally, fungus-treated and control residues were treated directly with 4 M NaOH at 170°C for 2 h to provide a measure of the total amount of phenolics assessable by this method and also to determine the amount of soluble lignin released by 1 M NaOH (i.e., the amount of substances of the

TABLE 2. Ratios of aromatic compounds in Bermuda grass stems after fungal treatment[a]

Fungal treatment	PCA:FA after treatment with:			G:S after treatment with:	
	1 M NaOH	4 M NaOH (sequential)	4 M NaOH (direct)	4 M NaOH (sequential)	4 M NaOH (direct)
None (untreated control)	2.63 ± 0 a	1.18 ± 0.04 a,b	1.19 ± 0.01 a	0.68 ± 0.01 a,b	0.53 ± 0 a,b
P. chrysosporium K-3	6.35 ± 0.30 d	1.14 ± 0.03 a,b	1.38 ± 0.01 b	0.88 ± 0.10 a	0.45 ± 0.03 c
P. chrysosporium K-3 mutant 3113	4.28 ± 0.23 c	1.03 ± 0.04 b	1.34 ± 0.03 b	0.63 ± 0.01 b	0.48 ± 0.03 b,c
P. chrysosporium K-3 mutant 85118-b	3.30 ± 0.54 a,b	1.22 ± 0.13 a	1.42 ± 0.11 b	0.72 ± 0.22 a,b	0.55 ± 0.04 a
P. pini RAB-83-19	3.69 ± 0.26 b,c	1.08 ± 0.07 a,b	1.32 ± 0.01 a,b	0.65 ± 0.01 a,b	0.45 ± 0.01 c
C. subvermispora FP 90031-sp	5.82 ± 0.15 d	1.10 ± 0.06 a,b	1.31 ± 0.09 a,b	0.60 ± 0.01 b	0.27 ± 0.01 d

[a] Average ± standard deviation of duplicate tubes. Values within columns with different postscript letters (a, b, c, and d) differ at $P \leq 0.05$. PCA, *p*-coumaric acid; FA, ferulic acid; G, guaiacyl units; S, syringyl units.

TABLE 3. In vitro dry matter loss of fungus-treated residues of Bermuda grass stems due to mixed ruminal microorganisms

Fungal treatment	% Dry wt loss after indicated time[a]			
	Inoculated tubes[b]			Noninoculated tubes (72 h)
	24 h	48 h	72 h	
None (untreated control)	19.8 ± 0.9 a	34.0 ± 1.2 b	34.4 ± 4.3 b	1.2 ± 0.1
P. chrysosporium K-3	41.7 ± 9.9 a	50.8 ± 7.4 a	46.9 ± 6.5 a	28.7 ± 5.4
P. chrysosporium K-3 mutant 3113	15.3 ± 4.2 a	30.5 ± 0.4 b	23.8 ± 3.2 a,b	11.8 ± 6.1
P. chrysosporium K-3 mutant 85118-b	38.1 ± 15.1 a	33.3 ± 4.7 a	34.4 ± 3.4 a	21.3 ± 30.0
P. pini RAB-83-19	19.9 ± 3.0 a	33.1 ± 2.4 b	35.3 ± 0.4 b	4.6 ± 0.3
C. subvermispora FP 90031-sp	41.9 ± 1.6 a	54.0 ± 2.3 b	63.9 ± 1.1 c	20.4 ± 1.8

[a] Average ± standard deviation of duplicate tubes.
[b] Digestion times. Values within rows with different postscript letters (a, b, and c) differ at $P \leq 0.05$.

guaiacyl and syringyl groups released by direct 4 M NaOH treatment minus the amount of those released by sequential 4 M NaOH treatment). Alkali-soluble constituents were prepared for evaluation by gas chromatography by acidification of the aqueous solution, extraction with diethyl ether, and silylation of the residue after removal of ether (26). Sinapic acid was used as an internal standard. To determine response factors for the individual constituents, authentic compounds were subjected to the reaction conditions followed by addition of the internal standard. The response factors used for quantification were 0.63 and 0.66 at 25°C and 0.62 and 0.96 at 170°C for *p*-coumaric acid and ferulic acid, respectively. Correction factors were as follows for the other compounds: vanillin, 0.94; acetovanillone, 0.78; syringaldehyde, 1.98; acetosyringone, 1.72; and syringic acid, 1.74.

In vitro digestibility and fermentation of Bermuda grass stem residues. Intact sections of stems were freeze-dried, weighed, and placed into Hungate tubes. Portions (5 ml) of prereduced basal medium (20) without carbohydrates were added to each of the tubes, which were then capped with rubber septa. Medium and stems (three sections per tube) were autoclaved and then inoculated with 0.4 ml of strained rumen fluid from a steer housed at the University of Georgia Dairy Research Center and fed primarily alfalfa hay. Duplicate tubes were inoculated for each of three digestion times, and duplicate tubes were left uninoculated to serve as washout controls for each treatment. Sections were retrieved after 24, 48, and 72 h of incubation at 39°C with ruminal microorganisms and after 72 h in uninoculated tubes. Plant material was freeze-dried, and dry weight loss due to microbial activity was calculated from initial dry weights. Ground plant residues were incubated in duplicate with ruminal microorganisms for 48 h, and volatile fatty acid concentrations were determined by gas chromatography as described elsewhere (37).

Transmission electron microscopy of Bermuda grass stem residues. Control and fungus-treated residues and similar stems incubated with rumen microorganisms for 72 h were prepared for transmission electron microscopy generally according to described procedures (4). Briefly, stems were fixed in glutaraldehyde and then OsO_4, and ultrathin sections of Spurr-embedded Bermuda grass were stained with uranyl acetate and lead citrate and then observed at 80 kV.

UV absorption microspectrophotometry of cell walls in Bermuda grass stem residues. Thin sections of intact stems and ground material were both evaluated for UV absorption microspectrophotometry. Untreated and fungus-treated stems were embedded in JB4-Plus resin and sectioned at a thickness of 4 μm for microspectrophotometry as described elsewhere (8). These sections were placed on quartz slides and mounted in glycerin under a quartz coverslip. Additionally, cells from the ground material were identified as parenchyma or nonparenchyma (i.e., sclerenchyma and vascular cells), and UV absorption spectra were obtained for the cell walls.

A computer-controlled Zeiss UMSP-80 microspectrophotometry system was used in the UV absorption mode, and spectra were collected, analyzed, and displayed with Lambda Scan software (6). Transmitted illumination was provided by a high-pressure xenon lamp (XBO 75 W) through a grating monochromator with a bandwidth of 5 nm. For thin sections, a 32× quartz lens, providing a final

TABLE 4. Volatile fatty acids produced during in vitro fermentation of fungus-treated residues of Bermuda grass stems by ruminal microorganisms

Fungal treatment	Amount[a] of indicated volatile fatty acid						Total amount[b] of acids
	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	
None (untreated control)	30.2 ± 1.3 a	9.8 ± 0.7 a	0.4 ± 0 a	6.1 ± 0.5 a	1.0 ± 0 a	0.5 ± 0 a	47.9
P. chrysosporium K-3	39.3 ± 2.4 b	13.8 ± 0.8 b	0.7 ± 0.1 c,d	8.0 ± 0.3 b	1.4 ± 0.2 a,b	0.7 ± 0.2 b,c	63.8
P. chrysosporium K-3 mutant 3113	29.2 ± 4.0 a	7.9 ± 1.0 c	0.5 ± 0.1 a,b	4.5 ± 0.5 c	1.2 ± 0 a,b	0.5 ± 0 a,b	43.9
P. chrysosporium K-3 mutant 85118-b	30.6 ± 0.7 a	10.3 ± 0.6 a	0.5 ± 0 a,b	5.4 ± 0.4 a,c	1.7 ± 0.5 a,b	0.6 ± 0.1 a,b,c	49.0
P. pini RAB-83-19	45.6 ± 6.1 b,c	12.9 ± 0.2 b	0.6 ± 0 b,c	7.9 ± 0.1 b	1.0 ± 0 a	0.6 ± 0 a,b,c	68.5
C. subvermispora FP 90031-sp	53.7 ± 2.3 c	19.7 ± 0.8 d	0.8 ± 0.1 d	9.4 ± 0.4 d	1.5 ± 0.3 a,b	0.8 ± 0.1 c	85.9

[a] In micromoles per milliliter (average ± standard deviation of duplicate tubes). Values within columns with different postscript letters (a, b, c, and d) differ at $P \leq 0.05$.
[b] In micromoles per milliliter.

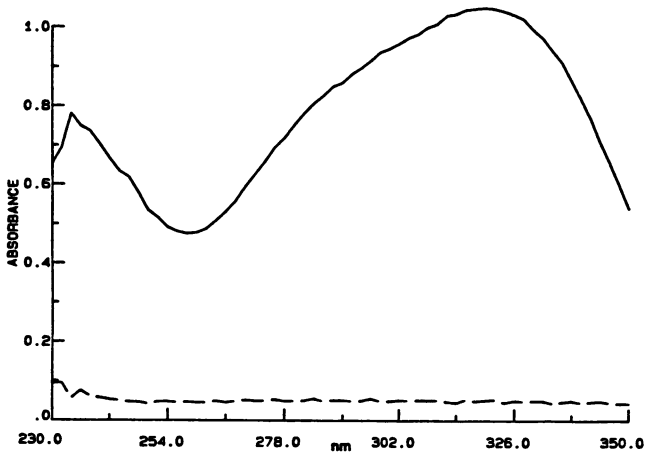

FIG. 1. UV absorption spectra of untreated cell wall (——) and of *C. subvermispora* FP 90031-sp-treated wall (– – –) of parenchyma in thin sections of Bermuda grass stem.

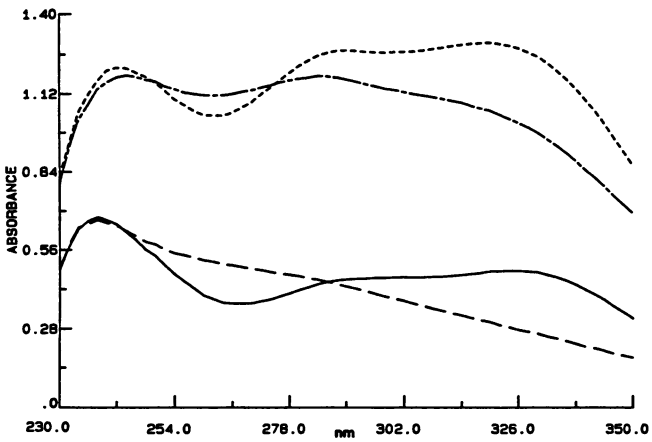

FIG. 3. UV absorption spectra of cell walls from ground Bermuda grass stems showing untreated parenchyma (——), *C. subvermispora* FP 90031-sp-treated parenchyma (— — —), untreated nonparenchyma (sclerenchyma and vascular cells) (-----), and *C. subvermispora* FP 90031-sp-treated nonparenchyma (— - — - — -).

aperture of 1.56 μm, was positioned over the cell walls or wall layers (i.e., secondary layer or middle lamella-primary layer). For cells in ground material, a 10× quartz lens, which provided a final aperture of 5 μm, was positioned over specific cell walls where the wall surface was smooth and uniform. The aperture was delimited within about 1/3 of the area of a field-limiting diaphragm to reduce stray light. Absorbance of transmitted UV illumination was measured from a range of 230 to 350 nm at 2-nm increments. The system was standardized at 350 nm.

Statistics. Values were analyzed by one-way analysis of variance using the least-square difference.

RESULTS

Aromatic constituents of residues. Sequential treatments with 1 M followed by 4 M NaOH and direct treatment with 4 M NaOH provided information on the distribution of various aromatic constituents and their linkages (Table 1) and on the relative proportions of components (Table 2). This method is reported to be an improvement over nitrobenzene oxidation for analysis of aromatic components (30), and assessment of it in other work (38) indicates that the constituents analyzed accounted for about 3% (wt/wt) of the cell wall or 8% (wt/wt) of the lignin. Substantial amounts of aromatics are not accounted for by this method. The ester-linked *p*-coumaric and ferulic acids released by 1 M NaOH were generally lower in fungus-treated residues than in untreated controls (Table 1). K-3 and FP 90031-sp removed these acids to a greater degree than the other fungal strains, with both acids and especially *p*-coumaric acid having the lowest levels in FP 90031-sp-treated residues. The *p*-coumaric acid/ferulic acid ratio was higher ($P < 0.05$) for K-3 and FP 90031-sp than that for other organisms or the control (Table 2), indicating that these two fungi were particularly able to remove ferulic acid. The statistic calculated as the difference of values from direct treatment minus those from sequential 4 M NaOH alkali treatment indicates that the levels of soluble lignin (i.e., guaiacyl and syringyl groups) released by the 1 M NaOH treatment were higher with FP 90031-sp treatment than with K-3 treatment or with untreated stems (Table 1). While differences in amounts of particular constituents were not generally significant ($P > 0.05$) in the sequential 4 M NaOH treatment, amounts of constituents in FP 90031-sp-treated residues were smaller than those for other fungal treatments (Table 1). Estimates of the total amounts of aromatics removed by alkali treatment (direct 4 M NaOH) were lower ($P \leq 0.05$) for K-3- and FP 90031-sp-treated residues, with estimates for residues treated with FP 90031-sp lower than those with all other treatments (Table 1). This observation indicated that treatment with these fungi had previously removed more aromatics than other treatments. The ratio of guaiacyl to syringyl was higher ($P < 0.05$) for K-3 than for FP 90031-sp in the sequential 4 M NaOH treatment (Table 2). For the direct 4 M NaOH treatment, FP 90031-sp-treated residue had the lowest ($P < 0.05$) guaiacyl:syringyl ratio, indicating a proportionately greater removal of guaiacyl groups by this fungus. Results suggest that FP 90031-sp treatment removed a greater proportion of guaiacyl groups than did treatment

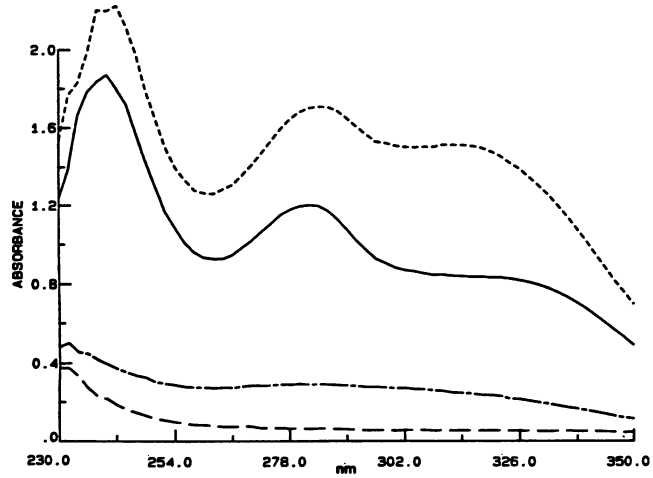

FIG. 2. UV absorption spectra of untreated secondary cell wall layer (——), untreated middle lamella-primary layer (-----), *C. subvermispora* FP 90031-sp-treated secondary layer (– – –), and *C. subvermispora* FP 90031-sp-treated middle-primary layer (— - — -) of sclerenchyma in thin sections of Bermuda grass stem.

TABLE 5. UV absorption microspectrophotometry of cell walls in fungus-treated residues of Bermuda grass stems

Cell type	Treatment	Stem no.	λ_{max} (nm) near 280 nm	A_{280}	λ_{max} (nm) near 320 nm	A_{320}	Ratio (A_{280}:A_{320})
Parenchyma	None (untreated control)	1	290 s[a]	0.860	320	1.049	0.82
		2	290 s	0.744	320	0.903	0.82
		3	290 s	0.382	320	0.474	0.81
	C. subvermispora FP 90031-sp	1	280	0.049	320	NA[b]	
		2	276	0.126	320	NA	
		3	276	0.187	320	NA	
Sclerenchyma secondary wall	None (untreated control)	1	282	1.201	322 s	0.832	1.44
		2	282	1.171	320 s	0.918	1.28
		3	282	1.729	320 s	1.144	1.51
	C. subvermispora 90031-sp	1	NA	NA	NA	NA	
		2	282	1.630	316 s	1.187	1.37
		3	NA	NA	NA	NA	
Sclerenchyma middle lamella region	None (untreated control)	1	284	1.708	314 s	1.511	1.13
		2	284	2.020	314	1.846	1.09
		3	286	2.332	314	2.079	1.12
	C. subvermispora 90031-sp	1	280	0.293	320 s	0.235	1.25
		2	282	1.860	316 s	1.423	1.31
		3	NA	NA	NA	NA	
Vascular bundle	None (untreated control)	1	280	1.617	322 s	0.983	1.64
		2	284	1.656	320 s	1.170	1.42
		3	284	1.733	314 s	1.339	1.29
	C. subvermispora 90031-sp	1	280	1.095	320 s	0.800	1.37
		2	284	1.478	316 s	1.177	1.26
		3	282	0.943	316 s	0.675	1.40

[a] s, shoulder.
[b] NA, no absorption maximum or shoulder.

with the other fungi and also resulted in a residue containing substantial amounts of alkali-soluble syringyl groups.

In vitro degradation by rumen microorganisms of fungus-treated residues. Dry weight loss due to mixed ruminal microorganisms after 24 h was greatest after treatment with K-3, 85118-b, and FP 90031-sp, although variation was high among replicate tubes containing residues from K-3 and 85118-b (Table 3). Dry weight loss increased during the period from 24 to 48 h for most cultures, indicating that fiber digestion occurred during this time. However, a significant increase in dry weight loss did not occur during the period from 24 to 48 h for K-3-treated residue. Levels of dry weight loss at 72 h were no higher ($P > 0.05$) than at earlier incubation times for residues, except for those from FP 90031-sp treatment. These results suggested that the full extent of in vitro digestion was obtained by this method, with the exception of that obtained with residues from FP 90031-sp treatment. In contrast, for residues from FP 90031-sp treatment the levels of dry-matter loss increased ($P < 0.05$) during the period from 48 to 72 h and were the highest ($P < 0.05$) levels of loss for all treatments, reaching 63.9% at 72 h for an increase of 80% over that for the untreated control. The levels of loss of dry matter in uninoculated tubes were highest for residues treated with K-3, 85118-b, and FP 90031-sp, with that for K-3 being the highest of all and the levels for replicate tubes from 85118-b being extremely variable (Table 3). The level of dry weight loss of stems treated with FP 90031-sp was 18% greater than that of untreated stems.

The levels of production of volatile fatty acids (Table 4) from fungus-treated Bermuda grass stems by rumen microbial fermentation in vitro were highest for residues treated with FP 90031-sp, and the levels of major acids, i.e., acetate, propionate, and butyrate, were significantly higher ($P < 0.05$) than those for the other residues. For analysis of fungus-treated residues at equal concentrations, the total production of 2-carbon to 6-carbon acids by ruminal microorganisms was 80% greater for FP 90031-sp-treated plant biomass than for untreated Bermuda grass stems.

Microscopic evaluation of fungus-treated residues. UV absorption microspectrophotometry of thin sections of untreated and FP 90031-sp-treated stems was carried out to characterize the aromatic constituents remaining in specific cell walls (Fig. 1 to 3; Table 5). For parenchyma cells (Fig. 1), untreated walls showed a pattern typical of ester-linked phenolic acids (6) as indicated by a shoulder near 290 nm and extended conjugation with a λ_{max} near 320 nm. This spectral pattern was pronounced in the middle lamella-primary layer region compared with the secondary layer (data not shown). Incubation with FP 90031-sp resulted in parenchyma walls visible by light microscopy, but with reduced contrast (not shown) and no UV absorption maxima (Fig. 1). This spectral pattern occurred for all three stems analyzed by this method (Table 5). For sclerenchyma walls of three FP 90031-sp-treated stems analyzed for UV absorption, two stems had no absorption and one showed little change from the untreated control (Table 5). UV spectra of walls showing loss of absorption are illustrated in Fig. 2. Aromatic constituents were removed by FP 90031-sp from both the secondary layer and the middle lamella-primary layer region. Secondary layers of the refractory walls of the vascular bundle showed little change after incubation with FP 90031-sp (Table 5).

For ground (1-mm) material, parenchyma cells were easily distinguished from nonparenchyma cells by light microscopy. As with thin sections, parenchyma walls treated with FP 90031-sp showed loss of UV absorption, with only a maximum near 240 nm (Fig. 3). In particular, absorption in the spectral regions indicative of ester-linked phenolics (i.e., near 320 nm) was absent. For lignified nonparenchyma walls (i.e., sclerenchyma and vascular tissues), FP 90031-sp reduced the UV absorption near 320 nm compared with

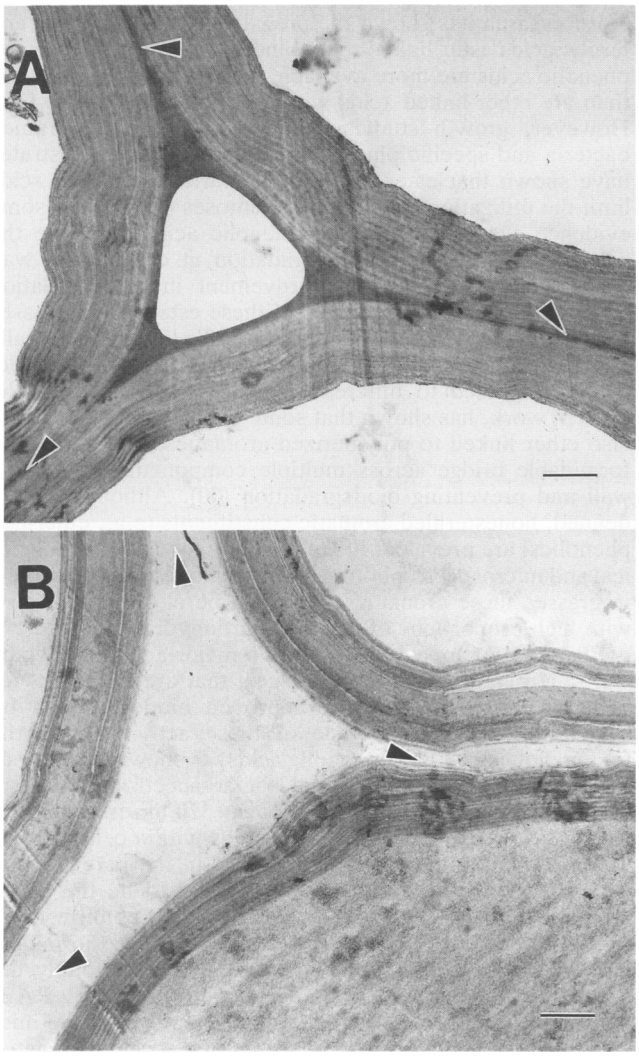

FIG. 4. Transmission electron micrographs of parenchyma cell walls from Bermuda grass stem fixed with glutaraldehyde and subsequently with OsO_4 and stained with uranyl acetate and lead citrate. Bar = 1 μm. (A) Untreated cell walls showing high electron denseness in the middle lamella region (arrowheads) and laminated cell walls. (B) *C. subvermispora* FP 90031-sp-treated cell walls showing overall loss of electron denseness, removal of middle lamella (arrowheads), and disruption of other wall components.

untreated control cell walls, suggesting particular removal of ester-linked aromatics by FP 90031-sp treatment.

Transmission electron microscopy was used to assess structural changes in walls of specific cells affected by FP 90031-sp. Untreated parenchyma walls (Fig. 4A) showed wall integrity with laminated walls and a greater electron denseness for middle lamella-primary layer regions. After treatment with FP 90031-sp (Fig. 4B), the middle lamellar region of parenchyma was electron transparent and cells were separated, indicating loss of wall material and tissue integrity. None of these cells remained after incubation with rumen microorganisms. The more recalcitrant sclerenchyma walls (Fig. 5) showed cell wall integrity and no loss of wall material in untreated controls or after digestion of untreated stems by ruminal microorganisms (Fig. 5A). After treatment with FP 90031-sp, the electron denseness of sclerenchyma walls was substantially modified (Fig. 5B), with regions which were adjacent to fungi and in which lignin loss apparently had occurred more electron dense; often, the secondary and middle lamella layers (arrowhead) were partially degraded. After FP 90031-sp treatment and incubation with rumen bacteria (Fig. 5C), considerable amounts of the secondary and middle lamella regions of sclerenchyma cell walls were eroded, resulting in substantial loss of wall material.

DISCUSSION

Results from analysis of fungus-treated residues for aromatic constituents, biodegradation by rumen microorganisms, and microscopic structural modifications all indicate that FP 90031-sp gave the best results. FP 90031-sp significantly removed aromatics, delignified plant cell walls, and improved ruminal microbial utilization of plant biomass. K-3 also improved the digestion over that observed for untreated control stems, but results within replicate tubes for dry weight loss were highly variable. Digestion values at 24 h were not significantly different from those of later incubation times. These marginal results, along with other data (5, 34) showing little or no improvement with this organism indicate that K-3 probably is not a suitable organism to improve biomass conversion of herbaceous material. The cellulaseless mutants developed from K-3 did not improve the utilization of lignocellulose by ruminal microorganisms, which supports other data for such mutants (5, 35). Our results indicated no improved fiber utilization by RAB-83-19, as was also observed in work by Jung et al. (33), despite the report of selective delignification of birch and pine by *P. pini* 2 (40).

Research on the lignin-degrading enzymes of FP 90031-sp has shown differences from K-3, and also has shown that manganese peroxidase and laccase are secreted during active growth (41, 42). FP 90031-sp has been reported to cause selective delignification with little weight loss in several wood species (2, 12, 40). Several strains of *C. subvermispora* caused selective lignin degradation of aspen, birch, and pine wood and moderate weight losses (12). Strain FP 90031-sp, which is the one tested in the present work, was superior to other strains in energy savings and fiber strength properties in pretreatment of pine chips (2). Our results in the present study further confirmed that *C. subvermispora* FP 09932-sp selectively delignifies plant cell walls and further indicated its value in treating grass lignocellulose for increased fodder value. Estimates of levels of dry matter loss in stems treated with FP 90031-sp were about 18% greater than those in untreated control stems. While extensive work was not performed on the carbohydrate moieties of the residues in this study, preliminary findings using spectroscopic analyses (28) suggested that FP 90031-sp did not reduce the wall polysaccharides as substantially as did K-3, but FP 90031-sp instead removed aromatics, thus exposing the carbohydrates for greater utilization by rumen microorganisms. Transmission electron microscopy of sclerenchyma cells indicated a greater electron denseness in wall components adjacent to fungal hyphae (Fig. 5B). Results from our studies and others (14), using white rot fungi and thin sections treated with OsO_4 and glutaraldehyde and uranyl acetate, suggest that lignin is removed selectively, allowing diffusion of heavy metals into remaining components of formerly lignified regions.

Aromatic constituents in grass cell walls consist of a diverse group of compounds with diverse linkages (19, 24,

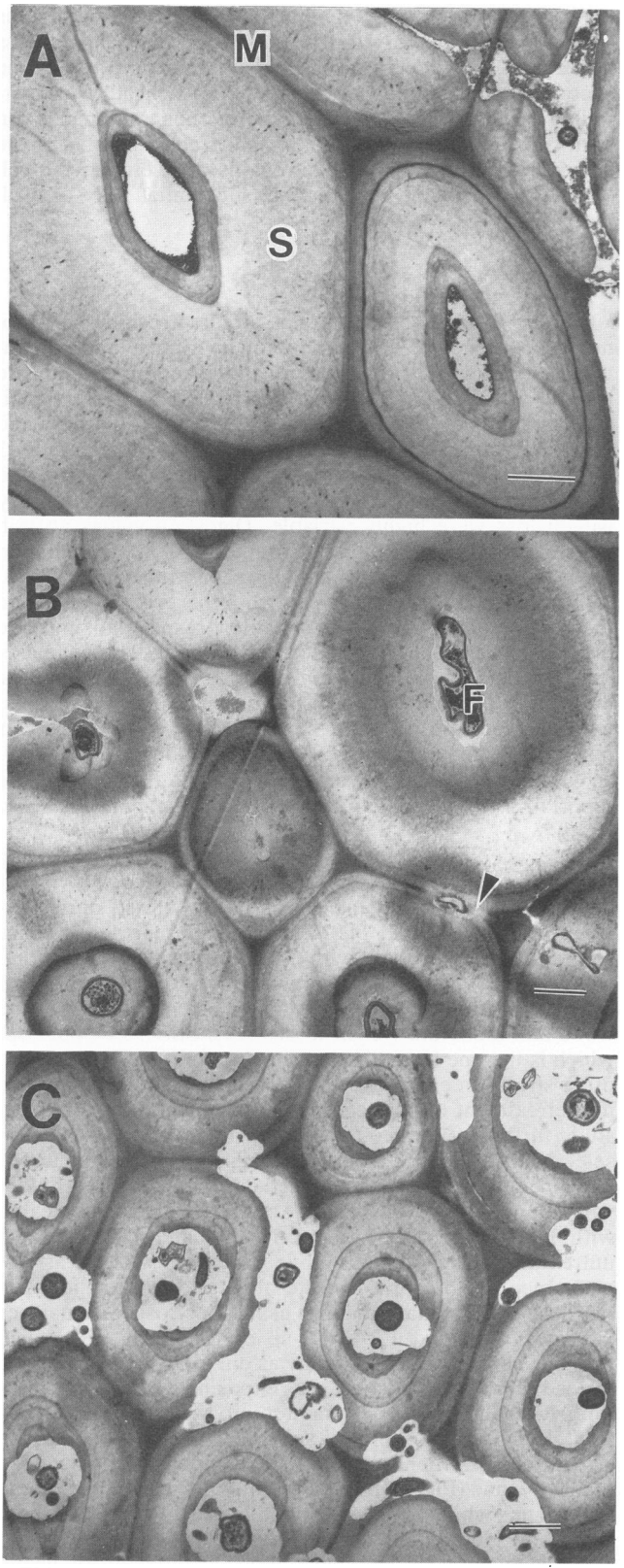

FIG. 5. Transmission electron micrographs of sclerenchyma cell walls from Bermuda grass stem fixed with glutaraldehyde and subsequently with OsO_4 and stained with uranyl acetate and lead citrate. Bar = 1 μm. (A) Untreated control incubated with rumen microorganisms in vitro showing resistance to biodegradation of secondary (S) and middle lamellar (M) layers. (B) Cell walls treated with *C. subvermispora* FP 90031-sp and not incubated with rumen microorganisms showing degradation of middle lamella (arrowhead) and fungi (F) in the cell lumen. Electron-dense regions, particularly in secondary walls, most likely indicate specific sites of lignin loss with greater staining by remaining carbohydrates. (c) Cell walls treated with *C. subvermispora* FP 90031-sp and incubated with rumen microorganisms in vitro showing loss of secondary layers and middle lamella and the presence of rumen bacteria throughout the tissue.

36). Research has shown the prevalence of *p*-coumaric and ferulic acids ester linked to arabinoxylans (24). Ester-linked phenolic acids are more available during ruminant digestion than are ether-linked acids within the plant cell walls (36). However, growth studies using pure cultures of rumen bacteria and specific phenolic acid-carbohydrate substrates have shown that ester-linked *p*-coumaric and ferulic acids limit the utilization of the bound pentoses (3). In fact, some evidence suggests that these phenolic acid esters are the primary impedance to biodegradation in certain cell wall types and that observed improvement in biodegradation arises from reduction in levels of these esters (26). Grasses appear to have a high level of esters in the lignified cell walls (8, 27). Treatment with the sequential alkali technique, which was used to differentiate esters and ethers in the present work, has shown that some phenolic acid esters are also ether linked to polymerized aromatics, thus forming a formidable bridge across multiple components of the cell wall and preventing biodegradation (30). Although still ill defined, nonesterified aromatic constituents (e.g., etherified phenolics) are prevalent in grasses, as shown by histochemical and microspectrophotometric techniques (6, 7). Further, in grasses these aromatics appear to be closely associated with high proportions of cell wall carbohydrates (29, 32).

FP 90031-sp appears to have two major activities, which were identified with our techniques, that are important for delignification and thus for improved biodegradation by rumen microorganisms. One of these activities was the removal of ester-linked phenolic acids, as shown by analysis using gas chromatography and microspectrophotometry. The reduction of UV absorption near 320 nm is consistent with loss of ester-linked phenolic acids within cell walls (6, 7). This effect is particularly apparent in the parenchyma, where ester-linked phenolic acids appear to be the major aromatic constituent (6). Such removal is confirmed by transmission electron microscopy showing that the middle lamella, which contains the most aromatics, is totally degraded by FP 90031-sp. A similar mode of attack by RAB-83-19 on birchwood, showing selective removal of the middle lamella, was observed (13). Esters that appear associated with more-condensed aromatic constituents, i.e., lignin, are also removed by FP 90031-sp, and this effect may facilitate biodegradation of the more recalcitrant cell walls such as those in sclerenchyma and vascular bundles. Agosin et al. (1) reported that esterified phenolic acids in wheat straw were rapidly degraded by three white rot fungi that preferentially removed lignin and improved digestibility. As in our results, they also found that ferulic acid was removed to a greater extent than *p*-coumaric acid. Some of the most detailed work on phenolic acid esterases has been carried out by Borneman et al. (16, 17) on the anaerobic fungi indigenous to the rumen. This work suggested that such esterases gave ruminal fungi an important advantage over ruminal bacteria (15) in attacking recalcitrant cell walls of grasses. That work, along with

our present findings, lends support to the hypothesis that phenolic acid esterases contribute to improving lignocellulose biodegradability.

A second activity by FP 90031-sp that is related to improved biodegradation is the attack on aromatics that are more rigidly bound, such as through ether linkages. These structures are more apparent in the recalcitrant cell types such as stem sclerenchyma and vascular tissue in older internodes (6). Agosin et al. (1) reported that improved digestibility by white rot fungi resulted from preferential degradation of β-O-4 ether-linked groups in wheat straw. Similarly, in degradation of wood by white rot fungi, Mulder et al. (39) showed attack on ether-linked lignin and greater removal of syringyl than guaiacyl units. It may be significant that in our study FP 90031-sp preferentially removed the guaiacyl units, which generally are more resistant to degradation by white rot fungi (1, 23, 39) than syringyl units. While the assumption is made that the proportions of groups analyzed reflect those in the entire aromatic complex, definitive conclusions concerning guaiacyl:syringyl ratios should await further analyses, such as might be provided on aromatic fingerprints by using pyrolysis mass spectrometry (39). In the cell walls most extensively affected by FP 90031-sp, microspectrophotometry indicated that lignin and other aromatics in secondary layers and middle lamella-primary layers were virtually all removed by FP 90031-sp. Therefore, our results suggest that the fungus attacked both ester- and ether-linked aromatics within the lignin complex.

To our knowledge, this is the first report that pretreatment with FP 90031-sp improves the biomass conversion of a grass, although other white rot fungi have shown promise in this regard. Specific sites and mechanisms related to improvement are suggested by the chemical and structural results. The improvement of 80% in the levels of dry weight loss and of volatile fatty acid production by ruminal microorganisms, with dry weight loss due to the fungus of only 18%, indicates potential for improving fodder value. Tests for toxicity of the organism and the treated biomass as well as for improved performance in large-scale animal trials are necessary to further evaluate the potential of this treatment.

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