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REVIEW

Structural and chemical properties of grass lignocelluloses related to conversion for biofuels

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Abstract Grass lignocelluloses, such as those in corn and switchgrass, are a major resource in the emerging cellulose-to-ethanol strategy for biofuels. The potential bioconversion of carbohydrates in this potential resource, however, is limited by the associated aromatic constituents within the grass fiber. These aromatics include both lignins, which are phenylpropanoid units of various types, and low-molecular weight phenolic acids. Structural and chemical studies over the years have identified the location and limitation to fiber degradation imposed by a variety of these aromatic barriers. For example, coniferyl lignin appears to be the most effective limitation to biodegradation, existing in xylem cells of vascular tissues. On the other hand, cell walls with syringyl lignin, e.g., leaf sclerenchyma, are often less recalcitrant. Ferulic and *p*-coumaric acids that are esterified to hemicellulosic sugars constitute a major limitation to biodegradation in non-lignified cell walls in grass fibers, especially warm season species. Non-chemical methods to improve bioconversion of the lignocelluloses through modification of aromatics include: (1) use of lignin-degrading white rot fungi, (2) pretreatment with phenolic acid esterases, and (3) plant breeding to modify cell wall aromatics. In addition to increased availability of carbohydrates for fermentation, separation and collection of aromatics could provide value-added co-products to improve the economics of bioconversion.

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Introduction

Currently, production of fuel ethanol depends almost entirely on corn grain. This industry, as well as the use of fuel ethanol itself, is rapidly expanding and beginning to increase prices of corn for feed and the downstream animal products. Cellulose as a substrate for ethanol has been of interest for several years, but recent events have called for a priority of research to commercialize the cellulose-to-ethanol biorefinery. Cost of lignocellulosic materials is lower than corn grain but processing for fermentation is more costly. The sugars in lignocellulose, which exist mostly as the polysaccharides cellulose and hemicellulose, are not readily available. Lignin and other aromatics covalently link with, and at times physically mask, plant carbohydrates, thus protecting these potential substrates from degradation [8, 18, 40]. Pretreatment is required to free the carbohydrates from aromatics for bioconversion to ethanol [51]. Most often, the suggested pretreatment is chemical.

A considerable body of knowledge is available from the animal nutrition discipline on barriers to bioconversion in plant fiber, and the same limiting factors exist for bioenergy concerns [8, 59]. Lignin, which is a complex of phenylpropanoid groups, is a common constituent of plant cell walls. Wood and dicotyledons have rigid, non-degradable lignified cell walls. Although lower than wood, grasses also have lignified cell walls, but their cell walls are also rich in low molecular weight phenolic acids ester-linked to arabinose [30, 40]. These low-molecular weight phenolic acids can form dimers and also form ether linkages to other aromatic constituents, thus posing a formidable barrier to bioconversion. Warm-season grasses, which include potential bioenergy crops such as corn stover, sugarcane (bagasse), bermudagrass, and switchgrass, are especially high in phenolic acid esters [4].

The objectives of this paper are to (1) review the structural/chemical barriers to bioconversion in grasses, especially warm-season grasses, (2) discuss environmentally friendly (non-chemical) strategies to reduce recalcitrance of grass lignocelluloses, and (3) identify potential co-products from the aromatic constituents of lignocellulose.

Structural and chemical factors influencing recalcitrance in grass lignocellulose

Biodegradation of specific tissue types

Response of plant substrates to strongly fibrolytic microorganisms within the cattle rumen shows differences in cell wall recalcitrance of various substrates and the influence of particular aromatic compounds. Figure 1 gives an example of the response to microbial degradation of different cell types in bermudagrass leaf blades and stems. Tissues staining for lignin with acid phloroglucinol (AP) have the most recalcitrant cell walls (Table 1). Other cell walls, i.e., epidermis and parenchyma bundle sheath (pbs) of leaf blades, are only partially degraded. Still other cell types, such as the thin-walled mesophyll cells between vascular bundles in leaves and immature stem parenchyma, are quickly and totally degraded.

Histochemical stains identify the location of particular lignin types within different cell walls that vary in degradation. A listing of several stains used to characterize cell types, the compound stained, and examples of cell walls that stain positive for these reactions are given in Table 1. Tissues that give a reaction with AP, as previously mentioned for the vascular bundles, have been shown to be the most recalcitrant in plants, including the grasses. Sarkanen and Ludwig [53] reported that AP "had universal application to all lignins, although the reaction may be weak or

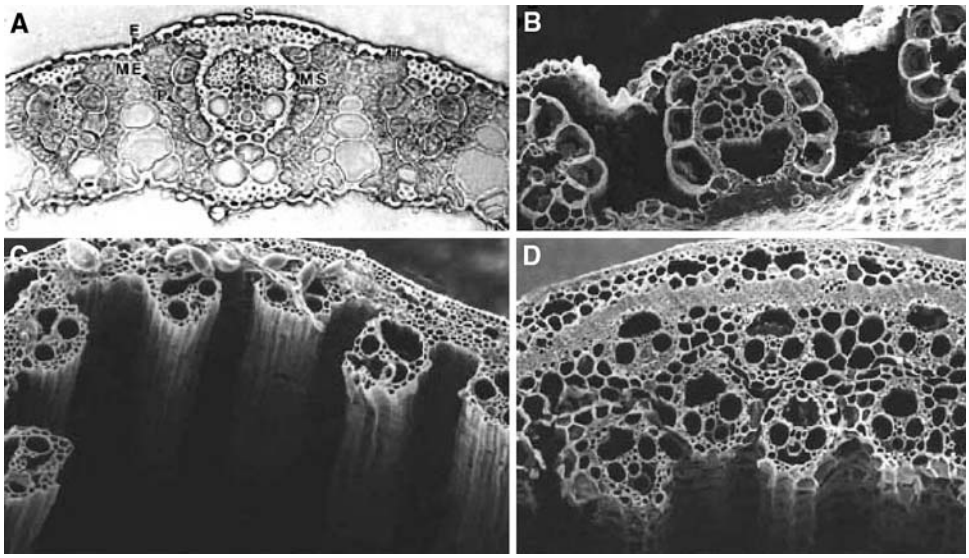

Fig. 1 **a** Light micrograph of cross section of untreated Coastal bermudagrass and identification of cell types: *E* epidermis, *S* sclerenchyma, *ME* mesophyll, *P* parenchyma bundle sheath, *MS* mestome sheath, and *PH* phloem. **b** Scanning electron micrograph showing disposition of cell types after incubation with rumen micro-organisms. Mesophyll and phloem are totally degraded, the epidermis and parenchyma bundle sheaths are partially degraded, and the sclerenchyma and mestome sheath are undegraded. **c** Scanning electron micrograph of immature stem after degradation showing the loss of parenchyma cells but residue of heavily lignified epidermis, sclerenchyma ring, and vascular tissues. **d** Scanning electron micrograph of more mature stem after degradation showing residue of parenchyma cells along with heavily lignified epidermis, sclerenchyma ring, and vascular tissues

Table 1 Histochemical stains for determining cell wall components in plant material

Stain	Compound	Examples	Reference
Acid phloroglucinol	Lignin (coniferyl)	Xylem cells	[53]
Chlorine-sulfite	Lignin (syringyl)	Leaf sclerenchyma	[53]
Diazotized sulfanilic acid	Phenolic constituents	Leaf parenchyma bundle sheath (warm-season grasses)	[7, 39]
Congo red	Cellulose	Non-lignified cell walls	[33]
Ruthenium red	Pectins	Middle lamellae	[33]
Oil red	Wax	Cuticle of epidermis	[16]

absent in lignins containing high amounts of syringyl propane units." The deep red to purple color reaction indicates a strong contribution to the cell walls by coniferyl (monomethoxylated) units of lignin. Leaf blade sclerenchyma, which is also poorly degraded, stains positive with chlorine water followed by sulfite (CS+) and indicates lignin rich in syringyl (dimethoxylated) units [53]. At times, CS+ tissues are partially degraded and are more susceptible to some chemical treatments, e.g., alkali, than AP+ tissues.

Warm-season grasses have non-lignified cell types, i.e., do not show a histochemical reaction for lignin with AP or CS, but nonetheless resist biodegradation. An example is shown for bermudagrass leaf blade in which the pbs and a portion of the epidermis is not degraded (Fig. 1b). It is worth repeating here that grasses, and particularly warm-season species, have high levels of ester-linked *p*-coumaric and ferulic within the cell walls [30, 40]. Use of diazotized sulfanilic acid indicates phenolic compounds in non-lignified, but still non-biodegradable, cell walls [39]. The positive histochemical reactions of pbs and epidermis in leaf and parenchyma in stem with diazotized sulfanilic acid suggest a prominent role for these ester-linked phenolic acids as a factor in the recalcitrance of grass lignocellulose [7].

Ultraviolet absorption micro-spectrophotometry (UV) provided further information on aromatics in cell wall types influencing biodegradation [6, 9, 41]. Examination of specific compounds, namely coniferyl lignin [56] and isolated ferulic acid ester-linked to arabinose linked to xylose units (FAXX) and similar structures but with *p*-coumaric acid (PAXX) [27], provided spectral information on specific plant-related, aromatic compounds. Along with spectral patterns of these individual compounds, spectral patterns in specific cell wall types are shown in Fig. 2. A λ max near 280 nm is typical for lignin (Fig. 2a). In contrast, both ester-linked phenolic acids show a bathochromic shift from 280 to a shoulder near 290 nm with λ max near 320 nm. The compounds FAXX and PAXX can be differentiated by λ max's near 326 and 314 nm, respectively (Fig. 2b). With absorbance for these compounds and compositional data on aromatic compounds in grasses, information was obtained from individual cell types and related to biodegradation and recalcitrance. Mestome sheaths of grasses all show prominent absorbances near 280 nm and near 320 nm (Fig. 2c). This suggests the presence of lignin and phenolic acid esters within these highly lignified (as shown with AP), non-degradable cell walls.

Parenchyma bundle sheaths are a prominent part of the Kranz anatomy of warm-season grasses and the C-4 dicarboxylic acid pathway of CO_2 fixation [54]. These cell walls show a UV spectral pattern identical to phenolic acid esters, suggesting little or no polymeric lignin but the presence of phenolic esters (Fig. 2c). The slow to partial degradation pattern of pbs in Coastal bermudagrass (Fig. 1b) coincides with the presence of phenolic acid esters. Further evidence of the presence of phenolic acid esters within cell types is shown by treating leaf blades with increasing concentra-

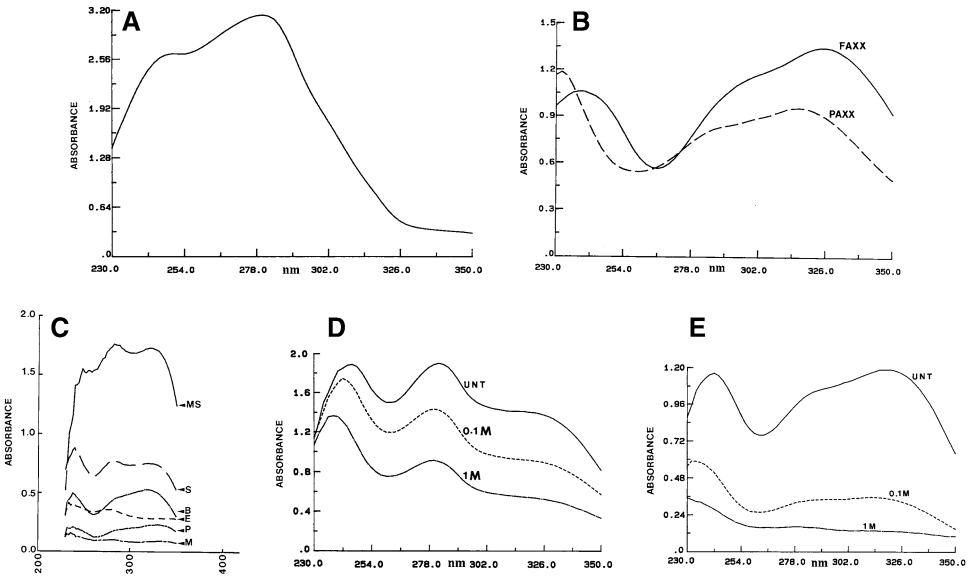

Fig. 2 UV absorption micro-spectrometry of aromatic compounds and cell walls of bermudagrass leaf blade. **a** Synthesized coniferyl lignin with a λ max near 280 nm and no absorbance beyond. **b** Isolated phenolic acid esters from bermudagrass. FAXX is ferulic acid esterified to arabinose and two connected xylose moieties. PAXX is similar to the compound above but with *p*-coumaric acid. These esters have a shoulder near 290 nm and a λ max near 326 nm for FAXX and 314 nm for PAXX. **c** Spectral patterns of cell walls in Coastal bermudagrass leaf blade: *MS* mestome sheath, *S* sclerenchyma, *B* parenchyma bundle sheath, *E* epidermis, *P* phloem, and *M* mesophyll. **d** Mestome sheath untreated and treated with 0.1 and 1 M NaOH showing preservation of UV absorbance near 280 nm but loss of absorbance near 320 nm. **e** Parenchyma bundle sheath showing loss of UV absorbance with 0.1 and 1.0 M NaOH. Adapted from Akin et al. [6], Akin and Hartley [9]

tions of NaOH, which breaks ester bonds [10]. In mestome sheaths, absorbance near 320 nm but not 280 nm is reduced (Fig 2d), suggesting that lignin remains, while phenolic acid esters are at least partially removed. In contrast, all UV absorption in the pbs is removed with alkali treatment (Fig. 2e), further supporting the idea that these esters exist as a barrier to biodegradation of these cell types.

Variation in this pattern of degradation exists for highly digestible cool-season grasses such as orchardgrass. While the lignified mestome sheath is not degraded in either bermudagrass or orchardgrass (Figs. 1b, 3a), the pbs in orchardgrass, in contrast to that in bermudagrass, is rapidly and completely degraded (Fig. 3a), even by the mildly fibrolytic rumen protozoa (Fig. 3b, c) [2, D. E. Akin and L. L. Rigsby, unpublished data]. In UV absorption spectra comparing bermudagrass and orchardgrass, the non-degraded mestome sheaths have similar UV absorption patterns, showing both lignified and esterified phenolic compounds [11]. In contrast, no UV absorption occurs in orchardgrass pbs. Rapid and complete degradation of the pbs, along with the lack of aromatics in the pbs shown by histochemical stains and UV absorption, further suggest the role of phenolic acid esters as a barrier to degradation in warm season grasses and that variations exist among grasses. Other work has shown that growth of fermentative rumen bacteria is limited when sugars are ester-linked to feruloyl and *p*-coumaroyl groups [12].

With the important attribute of esterified phenolic acids in non-lignified but undegraded grass cell walls, strategies to influence these compounds could be used to improve cell wall degradation. Some non-chemical means of removing or modifying phenolic acid esters and other aromatics have been evaluated in tissues to improve biodegradation [20].

Biological strategies to overcome recalcitrance

Pretreatment with lignin-degrading fungi

While chemical pretreatment has been proposed mostly to delignify lignocelluloses for improved bioconversion, there are other, environmentally friendly possibilities. White-rot fungi are recognized as the most active lignin-degrading micro-organisms [34]. Oxidative enzymes produced by the fungi, along with catalysts, produce free radicals in the aromatic moieties, which result in degradation of aromatic compounds. Well-publicized oxidative enzymes from white rot white include: laccases, manganese peroxidase, and lignin peroxidase. At least one of these enzymes, laccase with an activator, is commercially available.

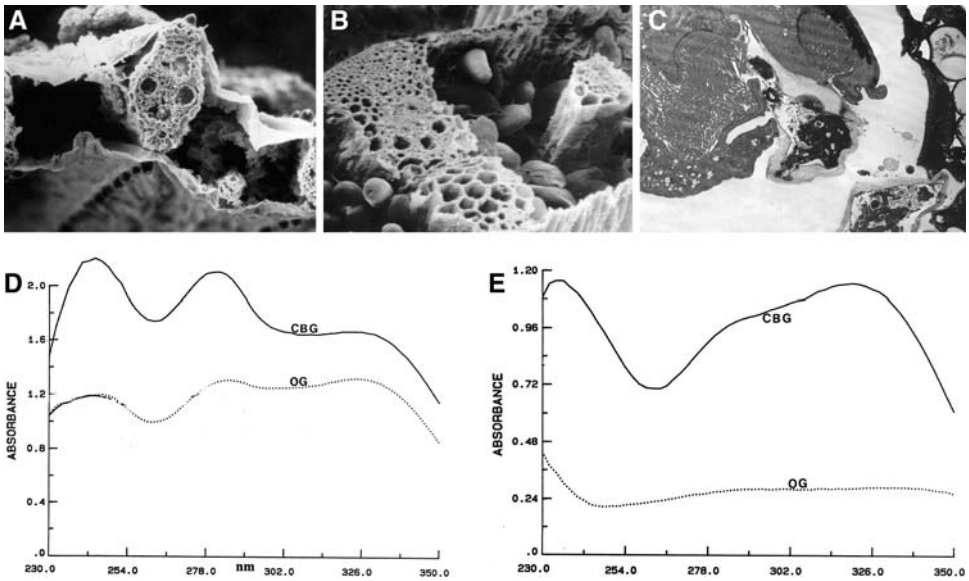

Fig. 3 Degradation of cell types in cool season leaf blade. **a** Scanning electron micrograph of orchardgrass leaf blade degraded with rumen micro-organisms showing resistant of lignified vascular bundle but total degradation of mesophyll, parenchyma bundle sheath (*pbs*), and epidermis. **b** Scanning electron micrograph of a leaf blade degraded by the weakly fibrolytic rumen protozoa, which are buried within the tissues. **c** Transmission electron micrograph of leaf blade showing partial degradation, separation and ingestion of a parenchyma bundle sheath cell wall by a protozoan. **d** UV absorption micro-spectrometry comparing the spectra of the non-degradable mestome sheath of Coastal bermudagrass (*CBG*) and orchargrass (*OG*). Both grasses have a λ max near 280 nm and a strong absorption near 320 nm, suggesting a lignified cell wall with esterified phenolic compounds. **f** UV absorption micro-spectrometry comparing the spectra of the parenchyma bundle sheath (*pbs*) of Coastal bermudagrass (*CBG*) and orchargrass (*OG*). The cell wall of a non-degraded pbs of CBG shows a strong absorption with a spectrum identical to that of phenolic acid esters, while the non-degraded cell wall of OG pbs, which is rapidly and completely degraded, has little to no UV absorption, indicating the absence of phenolic acid esters. From Amos and Akin [19], Akin and Amos [2], Akin and Rigsby [11]

A variety of white-rot fungi occurs in nature. They are recognized by large, colored fruiting bodies on dead and decaying trees (Fig. 4). Mycelia penetrate the woody cells, release a variety of enzymes, and produce a white delignified material. The ability of white rot fungi to delignify woody materials has been known for some time. Activities differ among genera and species and with various fungi-substrate combinations. The common pattern of attack on lignocellulose by these fungi is a simultaneous decay of polysaccharides (i.e., cellulose and hemicellulose) and lignin [34]. Patterns of decay and degrees of delignification, however, vary among species and even strains. Some species have the ability to selectively delignify plant material and leave a cellulose-rich residue [24]. Such a characteristic could be useful in providing an unprotected carbohydrate for subsequent use, e.g., animal feed or biofuel substrate. Toward this end, studies have been undertaken using different white-rot fungal species to improve forage utilization, but with mixed results [46]. Several species of white-rot fungi, emphasizing cellulase-less mutants and species reported to lack cellulase were evaluated to selectively attack lignin [13].

Data in Table 2 review results obtained with four white-rot fungi having different capablilities. *Phanerochaete chrysosporium* is a well-studied white-rot fungus that non-selectively attacks cell wall components, i.e., lignin and carbohydrates [34]. A mutant developed from this fungus, i.e., 3,113, reportedly lacks cellulase. *Phellinus pini* RAB-83-19 is reported to selectively degrade birch and pine wood [52]. *Ceriporiopsis subvermispora* lacks cellulase, produces manganese peroxide and laccase, and selectively delignifies several wood species [1]. Prior to our work, this fungus had not been evaluated for improvement of grass lignocellulose. Our data indicated that *C. subvermispora* was better able to attack the aromatics in bermudagrass and improved the utilization of pretreated residue over untreated material or residues pretreated by the other fungi tested [13]. *C. subvermispora* removed lignin units, preferentially attacking guaiacyl units over syringyl units, as well as ester-linked phenolic acids from unlignified cell walls,

Fig. 4 Examples of fruiting bodies of white rot fungi on decaying woody material. *White* rot fungi are the most effective microbial degraders of lignin, producing mycelia that penetrate plant cell walls and degrade various components

Table 2 Influence of pretreatment with species of white rot fungi on bioconversion of plant lignocellulose

	Characteristics of pretreated lignocellulose			
	Residual aromatics		Bioconversion potential[d]	
Fungus	1 M NaOH (mg g^{-1})	4 M NaOH (mg g^{-1})	DW loss (%)	VFA produced (μmoles ml^{-1})
Untreated	13.0	22.9	34.9	47.9
P. chrysosporium K-3[a]	7.1	18.9	46.9	63.8
P. chrysosporium m. 3113	11.9	25.0	23.8	43.9
Ph. pini RAB-83-19[b]	9.5	22.3	35.3	68.5
C. subvermispora 90031-sp[c]	5.3	17.8	63.9	85.9

[a] *Phanerochaete chrysosporium* is a well-known and studied white rot fungus that produces cellulases, hemicellulases, and lignin-degrading enzymes. M. 3113 is a cellulase-less mutant developed from *P. chrysosporium* K-3

[b] *Phellinus pini* reportedly selectively degrades some woody materials [52]

[c] *Ceriporiopsis subvermispora* is a white rot fungus that does not produce cellulase

[d] Dry weight (*DW*) loss if a measure of degradability. Volatile fatty acids (C-2 through C-6) are a measure of fermentability

Adapted from Akin et al. [13]

e.g., the pbs of bermudagrass (Table 2). Selective delignification of the middle lamella of stem parenchyma, while leaving the secondary wall, is shown in Fig. 5a, b. A more highly lignified structure, the sclerenchyma ring of stems (Fig. 1d), is also delignified and made available to subsequent bacterial degradation (Fig. 5c, d). That lignin is indeed selectively removed from these cell walls as shown by UV absorption spectra of untreated and fungal-treated cell walls (Fig. 5e, f).

A series of other white rot fungi and forage materials was evaluated in an expanded study [15]. Activity varied with the fungus, plant material, and the interaction of fungus and substrate. Two strains of *C. subvermispora*, i.e., CZ-3-8497 and FP-90031-sp, and *Cyathus stercoreus* were particularly effective in removing aromatics, especially ester-linked phenolic acids, and improving biodegradability.

The effectiveness of delignification by white rot fungi has been employed for several practical applications, including biopulping and improved forage quality. Little, however, seems to have done with white rot fungi for bioenergy. Keller et al. [46] reported preliminary results of a three- to fivefold increase in cellulose digestibility of corn stover after pretreatment with *C. stercoreus* and a ten- to 100-fold reduction in shear force after treatment with *P. chrysosporium*. In protocols for white rot fungi and delignification of biofuel resources, appropriate choices of fungi and substrates are required to optimize activity. Various strains of *C. subvermispora* are particularly effective at selectively removing lignin, leaving the cellulose for further uses. Therefore, *C. subvermispora* appears to be a good starting point for pretreating biofuels.

Pretreatment with phenolic acid esterase

It is well established that ester-linked p-coumaric and ferulic acids are prevalent in grasses and their relatives. These esters cause recalcitrance in non-lignified tissues and vary among tissues as well as plants. Further, improvement in biodegradation of forage grasses often involves changes or removal of these esters. Warm-season grasses sought for biofuels like corn, switchgrass, and bermudagrass generally contain more of these esters than cool-season grasses [4]. A corollary to the question of improvement of cell wall bioconversion with biological pretreatments is the fate of released phenolic acids. Depending on amounts, phenolic acids are toxic to micro-organisms and to enzymes [3, 25, 50, 55]. *p*-Coumaric acid is more toxic (effective at lower levels) than ferulic acid. Phenolic acids that are released by pretreatment, therefore, likely will require removal from the system for optimal activity by the subsequent activity of

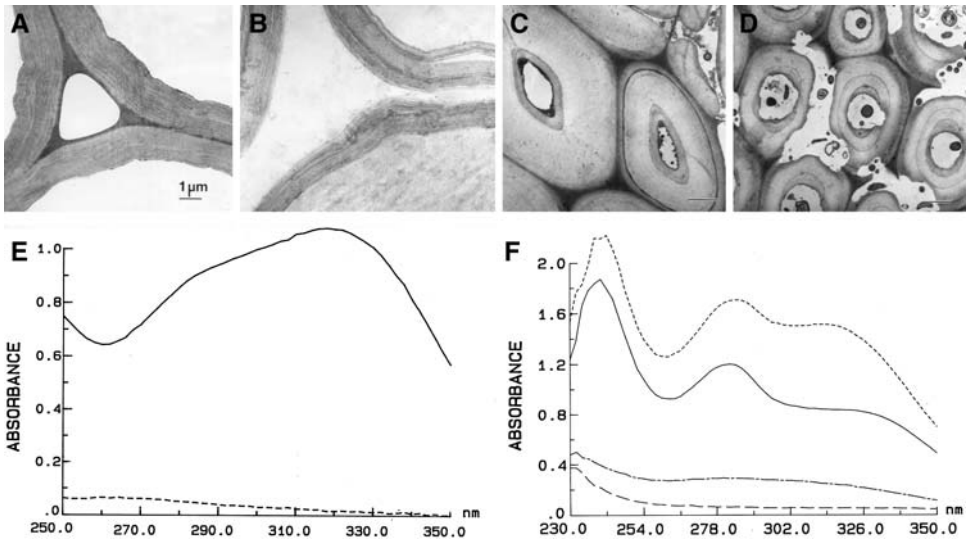

Fig. 5 Effect of pretreatment of bermudagrass stem with the *white* rot fungus *Ceriporiopsis subvermispora*. **a** Non-degraded, control parenchyma cell wall of stem showing intact middle lamella and secondary cell wall. **b** Stem parenchyma incubated with *C. subvermispora* showing loss of middle lamella but residue of secondary cell wall. **c** Heavily lignified sclerenchyma cell walls not treated by fungus but incubated with fibrolytic rumen micro-organisms showing resistance to degradation of cell walls. **d** Sclerenchyma cell walls pretreated with *C. subvermispora* and then incubated with fibrolytic micro-organisms showing bacterial degradation of middle lamellae and secondary wall material. **e** UV absorption spectra of stem parenchyma untreated (—) and incubated with *C. subvermispora* (– – –). Untreated cell walls have a spectral pattern indicative of ester-linked phenolic acids, whereas the fungal treated cell walls have no aromatics as shown by the loss of UV absorbance. **f** UV absorption spectra of lignified stem sclerenchyma: secondary wall incubated with fibrolytic micro-organisms only (—) and pretreated with *C. subvermispora* (— —); middle lamellae incubated with fibrolytic micro-organisms only (– – –) and pretreated with *C. subvermispora* (— – —). UV absorption does not occur after fungal treatment indicating loss of aromatics from remaining cell wall material. From Akin et al. [13]

enzymes and microbes (i.e., saccharification and fermentation). Evidence is strong that ferulic acid would be a valuable co-product from grass substrates and is discussed later.

Interest in our laboratory on phenolic acid esterases was initiated with discovery that anaerobic fungi within the rumen attack and weaken lignified cell types (Fig. 5). This activity appeared to be essential in certain feeding regimes in Australia (i.e., low quality, sulfur-fertilized warm-season grass) for animal growth [5]. Further work showed that these fungi preferentially attached to certain lignified tissues and were able to degrade lignified cell walls due to the presence of very active phenolic acid esterases, cellulases, and hemicellulases [26, 58]. A summary of work by Borneman indicates the ability of several isolates of anaerobic fungi to release *p*-coumaric and ferulic acids from bermudagrass cell walls [Table 3; 27].

With information on esterases and release of phenolic acids from grasses, work was initiated using commercial esterases to pretreat a series of potential warm-season grass substrates for biofuel [17, 20]. Other work confirms the value of cell-free ferulic acid esterases, along with hemicellulases, in releasing ferulic acid from a variety of plant materials [36, 47]. Our objective was twofold: to free carbohydrates for increased fermentation, and to release phenolic acids, especially ferulic acid, as value-added co-products. Initially, a series of studies with commercial esterases was carried out with a variety of warm-season grasses as potential biofuel sources. As expected, the use of ferulic acid esterase alone (i.e., cloned and without other cell wall-degrading enzymes) did not produce as high a biodegradation or as high a subsequent response to cellulase as mixed enzymes (unpublished data). Depol 740 L and TP692L, commercial mixtures containing ferulic acid esterase and hemicellulases, were tested at various levels and different conditions. Using dry weight loss as a marker for activity, a protocol was developed as follows: high doses of enzyme : substrate (1 g commercial product per 0.5 g ground substrate), incubation at pH 5.0 and 37°C with esterase for 24 h, removal of liquid, and re-incubation in cellulase at pH 5.0 and 37°C for 72 h. We found that a single incubation cocktail of Depol 740, an additional hemicellulase, and cellulase, were less effective for degradation than incubation with Depol 740 followed by sequential incubation with cellulase. The protocol was developed to maximize the activity of the enzymes and not, at this point, as the most cost efficient.

Table 3 Phenolic acids released by different anaerobic fungi

Culture filtrates[a] from:	Phenolic acids released from bermudagrass cultivars (μg 100 mg^{-1})	
	ρ-Coumaric acid	Ferulic acid
Neocallimastix MC-2	130	376
Piromyces MC-1	114	336
Anaeromyces PC-1	83	254
Orpinomyces PC-2	93	287
Orpinomyces PC-3	89	296

[a] Fungi were isolated from the rumen and characterized according to taxonomic criteria [58]

Adapted from Borneman et al. [26]

This protocol resulted in release of the simple sugars (arabinose, xylose, mannose, galactose, and glucose) from most all plant materials with each of the enzymes (i.e., phenolic acid esterase and cellulase. Phenolic acids released were virtually entirely *p*-coumaric and ferulic acids. Although Depol 740 is promoted as a ferulic acid esterase, substantial amounts of p-coumaric acid were also released, depending on composition of the substrate as shown below. Effective cellulose-to-ethanol protocols will require fermentation of all sugars. Therefore, xylose and glucose are reported as the predominant 5-carbon and 6-carbon sugars, respectively.

Diverse samples have been evaluated with this protocol (Table 4). In all samples, degradability by cellulase, as shown by % dry weight loss, was substantially increased by pretreatment with esterase. Esterase alone often released a substantial amount of phenolic compounds and sugars, with still more released with subsequent incubation with cellulase. An example of this effect by separate enzymes is shown in incubations of Tifton 85 bermudagrass leaf blades (Table 4).

A comparison of corn stover fractions, the above ground portion of the plant left after grain harvest, degraded by cellulase is shown with and without esterase pretreatment (Table 4). Dry weight loss and release of phenolic acids and sugars all improved with esterase pretreatment, but variations occurred with different fractions. Corn leaf fractions released the most glucose. Leaf sheath and stem pith released more ferulic acid than other fractions. For stems, the thinner-walled pith cells released more of all components than the thicker-walled, heavily lignified rind tissues. A second comparison was made of rind and pith tissues from a commercial source of corn stover (Table 4). These tissues were ground through a centrifugal mill to increase surface area and improve release of compounds. The corn stover source of these tissues was different from the previous ones, but results were similar in showing a higher level of *p*-coumaric acid than ferulic acid in stems and greater release with esterase treatment. These data indicate value in selecting portions of corn stover to collect for fermentation, leaving the least desirable fractions for the important attributes of soil carbon and erosion protection [57].

The brans of cereals offer the richest source for phenolic acids and can be released by enzymes from a variety of sources, such as corn [35], wheat [22], and oat [60]. The

Table 4 Dry weight loss, phenolic acids, xylose, and glucose from pretreatment of corn and bermdagrass lignocellulose plant components with Depol 740 ferulic acid esterase and cellulase

Sample	Enzyme treatment[a]	Dry weight loss (%)	*ρ*-Coumaric acid (mg g^{-1})	Ferulic acid (mg g^{-1})	Xylose (mg g^{-1})	Glucose (mg g^{-1})
Bermudagrass[b]	B	20.1 ± 0.2	0.16 ± 0.06	0.17 ± 0.02	0.5 ± 0.3	0.6 ± 0.1
	C	29.8 ± 0.7	0.19 ± 0.02	0.38 ± 0.02	8.0 ± 0.8	17.8 ± 5.3
	E	NA	0.37 ± 0.03	0.69 ± 0.05	17.6 ± 2.1	75.7 ± 4.7
	+C	47.2 ± 0.5	0.24 ± 0.05	0.33 ± 0.06	13.2 ± 2.0	65.6 ± 1.2
	E + C	47.2	0.61	1.02	30.8	141.3
Corn stover						
Leaf blade	C	40.9 ± 0.4	0.14 ± 0.02	0.33 ± 0.07	8.0 ± 0.7	53.0 ± 20.1
	E + C	61.8 ± 1.0	0.31 ± 0.06	0.58 ± 0.03	36.7 ± 3.5	125.0 ± 6.3
Leaf sheath	C	47.1 ± 1.3	0.09 ± 0.01	0.35 ± 0.02	11.1 ± 0.2	125.3 ± 99.1
	E + C	62.7 ± 1.8	0.52 ± 0.01	0.82 ± 0.03	39.0 ± 5.8	180.9 ± 11.8
Stem rind 1	C	16.4 ± 1.0	0.23 ± 0.14	0.10 ± 0.05	10.4 ± 0.6	39.2 ± 2.8
	E + C	20.5 ± 1.1	0.75 ± 0.04	0.39 ± 0.02	14.8 ± 1.8	34.5 ± 2.2
Stem pith 1	C	16.7 ± 0.7	0.26 ± 0.15	0.22 ± 0.11	23.0 ± 0.7	68.6 ± 1.5
	E + C	28.8 ± 0.7	0.95 ± 0.03	0.87 ± 0.3	34.0 ± 3.1	83.8 ± 15.4
Stem rind 2[c]	C	25.0 ± 0.4	0.55 ± 0.16	0.22 ± 0.08	10.7 ± 1.8	52.5 ± 13.2
	E + C	29.7 ± 1.9	1.39 ± 0.3	0.30 ± 0.13	22.5 ± 0.02	94.1 ± 2.1
Stem pith 2[c]	C	41.6 ± 1.8	0.58 ± 0.26	0.33 ± 0.17	20.2 ± 7.7	105.9 ± 36.2
	E + C	50.1 ± 3.0	1.90 ± 0.28	0.61 ± 0.09	49.6 ± 4.2	164.5 ± 5.6
Corn kernel fiber	B	7.1 ± 0.1	0.03 ± 0.01	0.03 ± 0.01	0	0
	C	26.5 ± 0.7	0.04 ± 0.01	0.05 ± 0.02	2.6 ± 0.4	36.7 ± 29.0
	E + C	48.2 ± 0.9	0.23 ± 0.03	2.7 ± 0.2c	8.2 ± 1.6	159.2 ± 3.0
	E + C[d]	56.9 ± 0.4	0.28 ± 0.01	3.5 ± 0.1b	12.1 ± 0.9	187.4 ± 24.9

[a] *B* buffer only 72 h, *C* cellulase only 72 h, *E* ferulic acid esterase (Depol 740L) 24 h, E + C sum of esterase followed by cellulase

[b] Tifton 85 bermudagrass blade

[c] Commercial corn stover hand-separated into rind and pith and ground through a Wiley mill with a 10 mesh and then 20 mesh screen and then ground through a centrifugal mill with a 0.8 mesh screen

[d] Ball-milled for a finer grind than other previous sample

Adapted from Akin et al. [17]

general structure of cereal grains is well known [14]. While the pericarp is more heavily lignified than other tissues, ester-linked phenolic acids predominate as the aromatic compounds in the aleurone layers. Corn fiber (i.e., the bran of the corn kernel that contains lignocellulose components) responded extremely well to ferulic acid esterase pretreatment [D. E. Akin and L. L. Rigsby, unpublished data]; giving the highest level of ferulic acid of substrates we tested (Table 4). Phenolic acid esterase extensively degraded the aleurone layer of corn fiber, but little structural change occurred in the pericarp. Relatively low levels of *p*-coumaric acid were released as low-amounts occur in corn fiber (less that 10% of phenolic acids analyzed by NaOH extraction). Grinding to finer particle sizes, using a laboratory ball-mill, led to high values for all parameters evaluated in this study, showing particularly high levels of ferulic acid and glucose (Table 4).

The use of esterases to release phenolic acids into the filtrate offers the possibility of a value-added co-product in processes of grass lignocellulose for bioconversion to ethanol. Since the phenolic acids are toxic to microbes and enzymes, the separation and collection of phenolic acids released by esterases may be necessary to optimize subsequent saccharification of pretreated materials. As an inhibitor of microbial growth and enzyme activity, these acids (especially *p*-coumaric acid) may have value as natural compounds for pest control [23]. Other potential uses for ferulic acid are a substrate for vanillin production, UV protection in cosmetics, and food antioxidants [37]. Future work should focus on the optimal enzyme cocktail (e.g., inclusion of appropriate hemicellulases), optimal substrates, physical treatments, and incubation conditions for the most cost efficient pretreatment system.

Plant breeding

Plant breeding to remove aromatics can result in plants extremely susceptible to pathogens and harsh conditions.

An extensive breeding program has existed for over 70 years in by ARS-USDA in Tifton, Georgia, and has been effective in producing sustainable new varieties of grasses, notably bermudagrasses, with improved biodegradability [38]. In many of these varieties, the improved biodegradability is related to lowered levels of aromatics. One notable example is Coastcross 1 bermudagrass (CC I) [28]. A study was conducted on CC 1 and one of the parents in the cross, namely Coastal bermudagrass (CBG) that is extensively grown as forage in the southern USA.

In direct comparison of the biodegradation of specific cell wall types, CC I was significantly higher in rate and extent of biodegradation compared with CBG (Table 5) [6]. Light and transmission electron micrographs of cell walls degraded by rumen bacteria indicated that non-lignified cell walls in CC I, while identical in structure to those in CBG, are considerably more biodegradable than those of CBG. UV absorption studies (Table 5) showed the mestome sheath cells, recalcitrant to biodegradation in both cultivars, had similar spectra. In contrast, pbs had less UV absorbance indicative of lower levels of phenolic acid esters in CC I, indicating that reduced levels of these compounds was at least one factor responsible for improved cell wall degradation.

Ferulic acid linkages between lignin and cell wall polysaccharides impede microbial break down of cell walls [44]. In the highly digestible bermudagrass Tifton 85 [29, 43], the ratio of ether- to ester-linked phenolic acids has been lowered, resulting in improved bioconversion [48, 49]. This work showed that Tifton 85 had higher neutral detergent fiber (NDF—an indication of total fiber content) and acid detergent fiber (ADF—an indication of lignified fiber) than Coastal bermudagrass, but both of these fractions had higher digestibility in Tifton 85. Although ester-linked ferulic acid levels were similar for 3- and 6-week old Tifton 85 (11.6 and 10.0 g kg^{-1}) and Coastal (10.6 and 10.6 g kg^{-1}), the ether-linked ferulic acid was lower for Tifton 85 (6.2 and 4.9 g kg^{-1}) than for Coastal (8.1 and 7.6 g kg^{-1}) [49]. In a similar study, Hatfield et al. [42] concluded the higher digestibility of Tifton 85 over Coastal bermudagrass was due to lower levels of lignin and cross-linked polysaccharides arising from lower amounts of ether-linked ferulates.

When bermudagrasses are pretreated with ferulic acid esterase and cellulase, both ferulic acid and *p*-coumaric acids are released. Although one study did not indicate significant differences in amounts of phenolic acids between genotypes (Table 6) [20], a second study supported the idea that Tifton 85 has higher ester linked phenolic acids than either Coastal or Coastcross II (CCII) (Table 7) [21]. Coastcross II is a mutant strain of Coastcross I and has been

Table 5 Comparison of cell wall biodegradability and chemical composition of Coastal versus Coastcross 1 bermudagrass

	Leaf dry wt. loss[a]			Area of pbs[a,b]	UV Absorbance[a]			
Cultivar	24 h	38 h	72 h	after 24 h	Parenchyma. bundle sheath		Mestome sheath	
	%			μm^2	λ max	A	λ max	A
Coastal	24a	36a	42a	350a	291	1.2a	285	1.8
					318	1.3a	318	1.8
Coastcross I	34b	49b	57b	196b	291	0.8b	288	2.0
					322	0.9b	321	1.9

[a] Different letters within a column indicate significant differences between cultivars ($P \leq 0.05$)

Adapted from Akin et al. [6]

Table 6 Percent dry weight (*DW*) loss, ferulic acid and free sugars released in filtrate after pretreatments with commercial esterase and cellulase and % in vitro dry matter digestibilities (*IVDMD*) using rumen fluids for bermudagrass genotypes at 4 and 8 weeks of age

Genotype	Age[a] (weeks)	% DW loss	Ferulic Acid (mg g^{-1})[b]	Glucose (mg g^{-1})[b]	IVDMD-Leaf	IVDMD-stem
Coastal	4	38.5 ± 0.4	1.1 ± 0.3	87.1 ± 6.7	46.01	46.18
Coastal	8	40.9 ± 0.2	0.9 ± 0.2	107.4 ± 4.2	39.75	49.25
Tifton 85	4	49.7 ± 0.5	1.3 ± 0.0	84.0 ± 13.3	58.53	58.74
Tifton 85	8	42.2 ± 0.6	0.8 ± 0.2	113.1 ± 2.7	54.84	50.17
Tifton 44	4	38.4 ± 0.4	0.8 ± 0.3	87.8 ± 1.8	50.43	53.61
Tifton 44	8	34.3 ± 0.4	0.8 ± 0.1	80.3 ± 0.9	51.62	45.21
CC II	4	45.5 ± 0.9	0.2 ± 0.1	111.7 ± 9.6	58.44	53.86
CC II	8	36.4 ± 0.0	1.0 ± 0.1	116.7 ± 2.4	51.96	43.27

[a] Plant age in weeks of regrowth

[b] Values are the sum of subsequent incubations with esterase for 24 h and then cellulase for 72 h

Adapted from Anderson et al. [20]

Table 7 Percent dry weight (*DW*) loss, ferulic acid, *para*-coumaric acid and free sugars released in filtrate after pretreatments with commercial esterase and cellulase for bermudagrass genotypes at 4 weeks of age

Genotype	% DW loss	Ferulic acid (mg g^{-1})	P-Coumaric acid (mg g^{-1})	Xylose (mg g^{-1})	Glucose (mg g^{-1})
Coastal	33.1cd	0.44 ± 0.04	0.31 ± 0.01	4.7 ± 0.1	84.0 ± 4.7
Tifton 85	41.8a	0.64 ± 0.02	0.46 ± 0.01	9.2 ± 0.4	112.2 ± 2.0
Tifton 44	32.2d	0.51 ± 0.11	0.37 ± 0.04	5.5 ± 2.5	78.5 ± 3.8
CC II	38.5b	0.44 ± 0.03	0.30 ± 0.01	6.1 ± 0.1	112.9 ± 4.1

Values are the sum of subsequent incubations with esterase for 24 h and then cellulase for 72 h

Adapted from Anderson et al. [21]

shown to be essentially the same in phenotype and genotype as Coastcross I (unpublished data). The Coastcross lines and Tifton 85 have consistently been similar and superior to Coastal bermudagrass in forage digestibility. The data also suggest this superior forage quality translates into higher conversion efficiency to sugars (Tables 6, 7) and ultimately to ethanol (Table 8).

Different genes, however, appear to determine the reduction of recalcitrance for these two bermudagrass cultivars. The Coastcross lines have lower levels of phenolic acid esters than Coastal, while Tifton 85 appears for have more ester-linked phenolic acids that are more easily released with esterases. The parents of the two cultivars are genetically dissimilar [28, 29], which is substantiated by molecular diversity data from amplified fragment-length polymorphisms (AFLP) analysis (unpublished).

Vogel and Jung [59] stated the importance of modifying plants for the use as a feedstock for biofuels. In addition to cellulose and lignin concentration changes, other traits that effect recalcitrance should be determined. From data presented on bermudagrass, it appears there is potential of increasing conversion efficiency to ethanol and isolating co-products through breeding of germplasm with different genes. Casler and Jung [32] found that breeding for improved digestibility is often accompanied by changes in lignin and levels of and linkages of phenolics in three perennial grass species. They suggest different strategies for selection for increased digestibility because of high correlations between neutral detergent fiber (NDF) and etherified ferulic acid. Switchgrass has been bred and selected for both high and low rumen digestibilities [31]. Sarath et al. (personal communication) investigated the alterations of the cell wall composition of selected plants within these populations. Other than a clear correlation between stem lignin and digestibility, plants varied greatly in the amounts of ester and ether linked phenolic acids, as well as guaiacyl and syringyl lignin within low and high lignin selections. They concluded that both the total lignin content as well as the extent of cross-linking will impact the DMD of plant tissues and influence the utility of specific genotypes as biofuel feedstocks. They also conclude the data indicates that levels of both *p*CA and FA had been significantly altered through selection for DMD at the whole plant level and suggest the potential to selectively breed for enzymatic release of each phenolic acid separately.

Table 8 Ethanol production and pentose residue of bermudagrass and switchgrass after dilute acid hydrolysis and simultaneous saccharification and fermentation (*SSF*)

Entry	Ethanol production (mg g^{-1})	Pentose (mg g^{-1})
Tifton 85	159.7a	182.8c
Coastcross II	156.5a	198.8b
Coastal	145.9b	171.0d
Switchgrass	116.2c	206.3a

Means with the same letter within a column are not significantly different ($P \leq 0.05$)

Courteously supplied by Bruce Dien (USDA-ARS, Peoria, IL, USA)

Increased knowledge of the genetic differences of cell wall components among specific cell types and among diverse grass germplasm is necessary to determine specific genes of interest. Together with the development of molecular maps associated with these traits for marker assisted selection (MAS), methods can be provided for genetic improvement of biomass species. Along with knowledge of genetic modifications, specific responses to bioconversion of individual cell wall types are required. Such information, derived from histochemical, microscopic, and micro-spectroscopic means, identify the cell wall modifications that actually affect cell wall bioconversion. It is clear from data presented above that tissues vary in bioconversion. Slight changes in highly lignified, recalcitrant cell walls may not result in improved bioconversion, whereas changes in non-lignified cell walls could substantially increase the availability of substrates, thus enhancing release of sugars and co-products. The challenge is to modify cell walls to optimize their potential as substrates in the cellulose-to-ethanol biorefinery.

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