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Introduction to the Proceedings of the Twenty-Eighth Symposium on Biotechnology for Fuels and Chemicals

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The Twenty-Eighth Symposium on Biotechnology for Fuels and Chemicals was held April 30-May 3, 2006 in Nashville, Tennessee which provided a new interesting venue as well as a convenient travel destination. The growing interest in alternative fuels and chemicals, driven in part by the rising cost of petroleum, along with the exciting technical program yielded a record attendance at this Symposium with 479 participants, exceeding late year's record attendance in Denver. Notable was a dramatic increase in representation from industry which comprised about 40% of the attendees, signifying the continuing transition of Symposium from biomass research to inclusion of commercial development and deployment. The Symposium continued to receive strong international support with 30% of the attendees coming from 24 counties. As always, the Symposium was a strong supporter of education as shown by the 86 students registered for the meeting at the reduced rate.

The 2006 28th Symposium on Biotechnology for Fuels and Chemicals marked a transition from a meeting organized and managed by the National Renewable Energy Laboratory and the Oak Ridge National Laboratory to transferring these critical duties to a professional not-for-profit scientific society, the Society for Industrial Microbiology (SIM). SIM took on the responsibility for contracting with the host hotel, handling registration, abstract submission and tracking, proceedings publication and overall management of the meeting. The technical aspects continue to be managed by the 21 member organizing committee whose responsibility is to support the meeting Co-Chairs. Session selection, abstract review, manuscript review and editing and proceedings editing is handled by the meeting Co-Chairs and overseen by the committee as it has been in previous years. This transition went very smoothly and the meeting was managed quite well by SIM even in the face of the record attendance.

Selection of the Charles D. Scott awardees marked a small but appropriate departure from tradition with selection of two equally deserving individuals based upon their significant, lengthy support of the Symposium both through their technical contribution for bioenergy research and also by their serving tirelessly as Co-Chairs for the Symposium for ten to twelve years! Brian Davison from Oak Ridge National Laboratory (ORNL) is the Chief Scientist for Systems Biology and Biotechnology. In his twenty years at ORNL he has performed biotechnology research in variety of areas including bioconversion of renewable resources, non-aqueous biocatalysis, systems analysis of microbes, and immobilization of microbes and enzymes among other areas of research. He supported the Symposium many ways before becoming co-chair of the 15th to 26th Symposium on Biotechnology for Fuels and Chemicals and served as editor of Proceedings in *Appl. Biochem. Biotechnol.*, (1994–2005). The Symposium grew from 150 to over 400 attendees during these twelve years (ten with Mark Finkelstein).

Mark Finkelstein joined the National Renewable Energy Laboratory (NREL) in 1992 to introduce industrial approaches to problem solving and helped establish numerous successful business relationships during his tenure at NREL. He helped initiate work on *Zymomonas* within the Biofuels Program that resulted in numerous patents, publications, NREL's first team Staff Award, and an R&D 100 award in 1995. He has served in a variety of capacities in support of the Symposium on Biotechnology for Fuels and Chemicals before becoming co-chair from 1996 to 2004. In 2004 Mark joined Luca Technologies, a small biotechnology company using microbes to create *in-situ* hydrogen/methane as a new source of energy. The Symposium on Biotechnology for Fuels and Chemicals congratulates both Brian and Mark for receiving the CD Scott Award in 2006.

These proceeding cover many of the 62 oral presentations and 255 poster presentations covering topics ranging from Feedstock Supply and Logistics, Microbial Catalysts and Metabolic Engineering, to Bioprocessing and Separations R&D, and Bio/Thermo-chemical Integrated Biorefinery. In addition, the Symposium hosted two special sessions on Life Cycle Analysis/Sustainability, and International Biomass/Biofuels Update.

Session Chairpersons

Session 1A: Enzyme Catalysis and Engineering

Chairs: Michael Himmel, *National Renewable Energy Laboratory, Golden, CO*
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Session 1B: Plant Biotechnology and Genomics

Chairs: Mariam Sticklen, *Michigan State University, East Lansing, MI*
Gerald Tuskan, *Oak Ridge National Laboratory, Oak Ridge, TN*

Session 2: Biomass Fractionation and Hydrolysis
Chairs: Amy Miranda, *USDOE Office of the Biomass Program, Washington, DC*
Charles Wyman, *University of California, Davis, CA*

Session 3A: New and Developing Industrial Bioproducts
Chairs: Mohammed Moniruzzaman, *BioEnergy Intl. LLC, Norwell, MA*
Christian Stevens, *Ghent University, Gent, Belgium*

Session 3B: Feedstock Supply and Logistics
Chairs: Robert Perlack, *Oak Ridge National Laboratory, Oak Ridge, TN*
Richard Hess, *Idaho National Laboratory, Idaho Falls, ID*

Session 4: Microbial Catalysis and Metabolic Engineering
Chairs: Stanley Bower, *Tate & Lyle, Decatur, IL*
Mark Eiteman, *University of Georgia, Athens, GA*

Session 5: Bioprocessing and Separations R&D
Chairs: Luca Zullo, *Cargill, Minneapolis, MN*
Seth Synder, *Argonne National Laboratory, Argonne, IL*

Session 6: Bio/Thermo-chemical Integrated Biorefinery
Chairs: Art Ragauskas, *Georgia Institute of Technology, Atlanta, GA*
Thomas Foust, *National Renewable Energy Laboratory, Golden, CO*

Special Topics Session A: Life Cycle Analysis/ Sustainability
Chairs: Bruce Dale, *Michigan State University, East Lansing, MI*
Michael Wang, *Argonne National Laboratory, Argonne, IL*

Special Topics Session B: International Biomass/Biofuels Update
Chairs: Jin-Ho Seo, *Seoul University, Seoul, Korea*
Barbel Hahn-Hagerdal, *Lund University, Lund, Sweden*

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Acknowledgments

The 28th Symposium was a success and these Proceedings are available due to the hard work of many people at ORNL, NREL and SIM in addition to the significant contributions of the Co-Chairs. In particular, Ann Luffman and Deneice Daniels at ORNL, Jim Duffield at NREL provided significant help to the Symposium and Proceedings. This author is particularly grateful for the diligent work of Christine Lowe and Demetra Pavidis at SIM for undertaking the difficult task of running the 28th Symposium the first time as it was underway and operating the meeting very successfully and Tracy Catanese at Humana Press for surviving through our delays.

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Other Proceedings in this Series

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3. "Proceedings of the Third Symposium on Biotechnology in Energy Production and Conservation" (1981), *Biotechnol. Bioeng. Symp.* **11.**
4. "Proceedings of the Fourth Symposium on Biotechnology in Energy Production and Conservation" (1982), *Biotechnol. Bioeng. Symp.* **12.**
5. "Proceedings of the Fifth Symposium on Biotechnology for Fuels and Chemicals" (1983), *Biotechnol. Bioeng. Symp.* **13.**
6. "Proceedings of the Sixth Symposium on Biotechnology for Fuels and Chemicals" (1984), *Biotechnol. Bioeng. Symp.* **14.**
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9. "Proceedings of the Ninth Symposium on Biotechnology for Fuels and Chemicals" (1988), *Appl. Biochem. Biotechnol.* **17,18.**
10. "Proceedings of the Tenth Symposium on Biotechnology for Fuels and Chemicals" (1989), *Appl. Biochem. Biotechnol.* **20,21.**
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13. "Proceedings of the Thirteenth Symposium on Biotechnology for Fuels and Chemicals" (1992), *Appl. Biochem. Biotechnol.* **34,35.**
14. "Proceedings of the Fourteenth Symposium on Biotechnology for Fuels and Chemicals" (1993), *Appl. Biochem. Biotechnol.* **39,40.**
15. "Proceedings of the Fifteenth Symposium on Biotechnology for Fuels and Chemicals" (1994), *Appl. Biochem. Biotechnol.* **45,46.**
16. "Proceedings of the Sixteenth Symposium on Biotechnology for Fuels and Chemicals" (1995), *Appl. Biochem. Biotechnol.* **51,52.**
17. "Proceedings of the Seventeenth Symposium on Biotechnology for Fuels and Chemicals" (1996), *Appl. Biochem. Biotechnol.* **57,58.**
18. "Proceedings of the Eighteenth Symposium on Biotechnology for Fuels and Chemicals" (1997), *Appl. Biochem. Biotechnol.* **63–65.**
19. "Proceedings of the Nineteenth Symposium on Biotechnology for Fuels and Chemicals" (1998), *Appl. Biochem. Biotechnol.* **70–72.**
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22. "Proceedings of the Twenty-Second Symposium on Biotechnology for Fuels and Chemicals" (2001), *Appl. Biochem. Biotechnol.* **91–93.**
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26. "Proceedings of the Twenty-Sixth Symposium on Biotechnology for Fuels and Chemicals" (2005), *Appl. Biochem. Biotechnol.* **121–124.**
27. "Proceedings of the Twenty-Seventh Symposium on Biotechnology for Fuels and Chemicals" (2006), *Appl. Biochem. Biotechnol.* **129–132.**

This symposium has been held annually since 1978. We are pleased to have the proceedings of the Twenty-Seventh Symposium currently published in this special issue to continue the tradition of providing a record of the contributions made.

The Twenty-Eighth Symposium will be April 30–May 3, 2006 in Nashville, Tennessee. More information on the 27th and 28th Symposia is available at the following websites: [http://www.eere.energy.gov/biomass/biotech_symposium/] and [http://www.simhq.org/html/meetings/]. We encourage comments or discussions relevant to the format or content of the meeting.

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SESSION 1A
Enzyme Catalysis and Engineering

Introduction to Session 1A

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Understanding and overcoming the natural resistance of plant cell walls to enzymatic hydrolysis remains one of the most active research areas in biofuels production (as indicated by the number of abstracts and papers submitted to this session). A number of the oral presentations given during the Enzyme Catalysis and Engineering session highlighted the use of new and innovative tools for advancing our understanding of plant cell wall deconstruction. The oral presentations and posters given for this session included applications of imaging tools and computational models to advance our understanding of biomass recalcitrance relative to enzymatic deconstruction. This session was opened with a presentation by Dr. Danny Akin, who outlined the structural and chemical barriers for the bioconversion of grasses to sugars. Lignocelluloses from grasses, such as switch grass, are resistant to bioconversion by various aromatic constituents, which include both lignins and phenolic acid esters. However, Akin and coworkers demonstrated the use of selected white rot fungal enzymes, which lack cellulases that could be used to produce delignified lignocellulosic materials, resulting in improved bioconversion.

Dr. Shi-You Ding presented an exposé on the use of new imaging tools now available at the National Renewable Energy Laboratory. Dr. Ding presented state-of-the-art applications of imaging and how it can be used to understand how plant cell wall microfibril structure changes during biomass conversion processes. Researchers now have the ability to

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examine the microfibril at the nanometer scale using atomic force microscopy, which allows for the imaging of biomaterials at atomic scale without extensive sample preparation or change in the original structure. Dr. Ding described how he has used this technique to visualize cellulases directly under aqueous conditions. His findings have resulted in a new model of the molecular structure of the cellulose microfibril in the plant cell wall.

Other presentations were focused on techniques to reduce biomass recalcitrance by applying thermal tolerant enzymes, supplementation with "accessory enzymes," or by using blocking agents, such as bovine serum albumin to prevent nonspecific absorption of enzymes to lignin. Dr. Deidre Willies from the Thayer School of Engineering at Dartmouth College presented data indicating that corn stover and lignin adsorb large amounts of cellulases and outlined methods that could be used to present adsorption and enhance cellulose digestion.

The presentations and resulting discussion during the session highlighted the need for more research that will advance our understanding of the natural resistance of plant cell walls to microbial deconstruction. It is this property that is largely responsible for the high cost of lignocellulose conversion; and yet to take the steps toward sustainable energy use we must overcome the chemical and structural properties that have evolved in biomass to prevent its deconstruction.

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Grass Lignocellulose

Strategies to Overcome Recalcitrance

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Abstract

Grass lignocelluloses are limited in bioconversion by aromatic constituents, which include both lignins and phenolic acids esters. Histochemistry, ultraviolet absorption microspectrophotometry, and response to microorganisms and specific enzymes have been used to determine the significance of aromatics toward recalcitrance. Coniferyl lignin appears to be the most effective limitation to biodegradation, existing in xylem cells of vascular tissues; cell walls with syringyl lignin, for example, leaf sclerenchyma, are less recalcitrant. Esterified phenolic acids, i.e., ferulic and *p*-coumaric acids, often constitute a major chemical limitation in nonlignified cell walls to biodegradation in grasses, especially warm-season species. Methods to improve biodegradability through modification of aromatics include: plant breeding, use of lignin-degrading white-rot fungi, and addition of esterases. Plant breeding for new cultivars has been especially effective for nutritionally improved forages, for example, bermudagrasses. In laboratory studies, selective white-rot fungi that lack cellulases delignified the lignocellulosic materials and improved fermentation of residual carbohydrates. Phenolic acid esterases released *p*-coumaric and ferulic acids for potential coproducts, improved the available sugars for fermentation, and improved biodegradation. The separation and removal of the aromatic components for coproducts, while enhancing the availability of sugars for bioconversion, could improve the economics of bioconversion.

Index Entries: Lignin; microspectrophotometry; phenolic acid esters; plant breeding; white-rot fungi.

Introduction

Corn-to-ethanol production and use is rapidly expanding. With the phase-out of methyl tertiary butyl ether, which has been used as an oxygenate for more efficient burning of gasoline, ethanol has been added in ever increasing quantities. Further, the desire to use even higher ratios of ethanol as a fuel related to improved national security, trade imbalance,

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and use of agricultural products has driven up the demand, price, and production. With greater emphasis on fuel ethanol, lignocellulose as substrate for fermentation has been given an increased priority (www.ethanolrfa.org) *(1)*. Recently, the Iogen Corporation reported the first commercial batch of ethanol from lignocellulose, using wheatstraw as a substrate (www.iogen.ca).

Cost of lignocellulosic materials is lower than corn grain, but processing for fermentation of this substrate is more costly. The sugars in lignocellulose, i.e., largely polysaccharides such as cellulose and hemicellulose, are not readily available, and pretreatment is necessary to free polysaccharides from lignin and aromatics *(2)*. Dilute sulfuric acid has been proposed for pretreatment by the United States Department of Energy, and this method has been tested in several variations. After pretreatment, enzymatic saccharification of polysaccharides to fermentable sugars is required. Finally, most lignocellulosic materials are rich in xylans and hemicelluloses, and 5-carbon fermentation is needed to take advantage of all the potential substrates *(3)*. Just as distillers dry grains and solubles as a value-added coproduct drives the economics of corn-to-ethanol, high-value coproducts are needed for lignocellulose-to-ethanol processes.

Lignin and other aromatics covalently link with, and at times physically mask, the potential fermentation substrates in lignocelluloses, thus protecting the carbohydrates from degradation *(4–6)*. Pretreatment, therefore, is required for bioconversion of lignocellulosic materials. A considerable body of knowledge is available from the animal nutrition discipline, and indeed many of the same limiting factors are important in bioenergy concerns *(6)*. Whereas woody plants and dicotyledons have rigid, nondegradable lignified cell walls, monocotyledons (e.g., grasses) have lignified cell walls as well as walls rich in low molecular weight phenolic acids, ester-linked to arabinose *(5)*. Another remarkable feature of grasses is that ester-linked *p*-coumaric and ferulic acids occur in nonlignified cell walls *(7)*. Warm-season grasses, which include potential bioenergy crops such as corn stover, sugarcane (bagasse), bermudagrass, and switchgrass, are especially high in the phenolic acid esters *(8)*. Because of the complexities within cell wall types, delineation of the nature, type, and location of aromatics within cell types of specific bioenergy crops can lead to environmentally friendly pretreatments, more efficient bioconversion, and identification of potential coproducts for high-value applications. The objectives of this article are to:

1. Review the structural/chemical barriers to bioconversion in grasses, especially warm-season grasses.
2. Discuss environmentally friendly (nonchemical) strategies to reduce recalcitrance of grass lignocelluloses.
3. Identify potential coproducts.

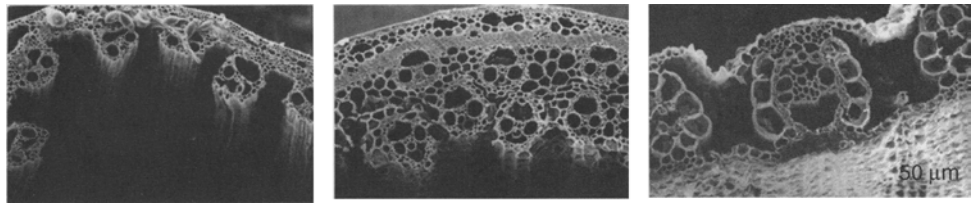

Fig. 1. Scanning electron micrographs showing biodegradation of grass fractions. Left—young stem showing resistance of epidermis, sclerenchyma ring, and vascular tissue with other cell types degraded. Center—mature stem showing resistance of epidermis, sclerenchyma ring, vascular tissue, and most of parenchyma cells; parenchyma nearest the stem center is partially degraded. Right—leaf blade showing resistance of vascular bundles, sclerenchyma, and portions of PBS; in addition to partial degradation of parenchyma bundle sheath and epidermis, mesophyll between vascular bundles and phloem cell walls are degraded.

Structural and Chemical Factors Influencing Recalcitrance in Grass Lignocellulose

Biodegradation of Specific Tissue Types

Response to the actions of fiber-digesting microorganisms within the cattle rumen shows differential cell wall recalcitrance within various lignocellulosic substrates and the influence of specific compounds. Figure 1 indicates the response of cell types in leaf blades and young and old stem internodes of a warm-season grass. The cell walls in the stems of Fig. 1 that resist biodegradation are many of the typically highly lignified cells. Walls are thick and cell contents are lacking, indicating a nonliving support tissue. Such cell types are thought to be the most resistant to degradation owing to the interaction of polymerized phenylpropanoid units with other consistuents. Histochemical stains identify the location of particular lignin types. Vascular cell walls, such as the mestome sheath, show a strong reaction with acid phloroglucinol (AP). Sarkanen and Ludwig *(9)* reported that AP "had universal application to all lignins, although the reaction may be weak or absent in lignins containing high amounts of syringyl propane units." Clifford *(10)* reported that various formulations of AP all detected most aldehydes, with various color responses for different aldehydes and those of cinnamaldehyde compounds giving a purple color. The deep red to purple color is taken to indicate a strong contribution to the cell walls by coniferyl (monomethoxylated) units of lignin. AP positive reactions (AP+) occur in vascular tissues of leaves and stems of grasses and these tissues, as indicated in Fig. 1, have been shown to be the most recalcitrant in grasses *(11)*.

Chlorine water followed by sulfite (CS) is reported to indicate lignin containing large amounts of syringyl (dimethoxylated) units of lignin *(9)*. CS+ reactions occur in leaf blade sclerenchyma (extensions of the vascular bundles) and in the parenchyma cell walls of mature (but not immature)

Table 1
Histochemical Reactions for Lignin and Relative Biodegradation of Cell Walls in Grass Lignocelluloses

Cell wall type	Histochemical reaction[a]	Area[b] (%)	Biodegradation[c]
Leaf blade			
Xylem/mestome sheath	AP	4 ± 2	None
Sclerenchyma	CS	6 ± 3	Slow to partial
Epidermis	–	35 ± 10[d]	Slow to partial[d]
	–	22 ± 6[e]	Rapid[e]
Parenchyma bundle sheath	CS	15 ± 7[d]	Slow to partial[d]
		6 ± 2[e]	Rapid[e]
Mesophyll	–	38 ± 9[d]	Rapid
	–	57 ± 5[e]	Rapid
Stem[f]			
Epidermis + sclerenchyma	AP	34 ± 4	None
Ring + vascular (xylem)	–	–	–
Parenchyma (mature)	CS	55 ± 6	Slow to partial
Parenchyma (immature)	–	–	Rapid

From ref. *11*.
[a]Most dominant staining reaction. No designation means histochemical reaction not prominent.
[b]Calculated from morphometric determinations.
[c]Response to fiber-degrading, rumen microorganisms.
[d]Warm-season.
[e]Cool-season.
[f]Warm-season.

stems *(11,12)*. The mechanism for reaction is unknown, and at times nonlignified cell walls show a positive reaction with chlorine-sulfite, suggesting that compounds other than syringyl lignin react. At times, CS+ tissues are partially degraded and are more susceptible to some chemical treatments, for example, alkali, than AP+ tissues. Table 1 compares many features in cell types of warm- and cool-season grasses related to biodegradation.

Often, and particularly in warm-season grasses, nonlignified cell types that do not show a histochemical reaction for lignin resist biodegradation. An example is shown for bermudagrass leaf blade in which the parenchyma bundle sheath and a portion of the epidermis is not degraded (Fig. 1). It is well established that grasses, and particularly warm-season species, have high levels of ester-linked *p*-coumaric and ferulic within the cell walls *(6,8)*. Use of diazotized sulfanilic acid to show phenolic compounds

Table 2
UV Absorption of Phenolic Esters

	λ_{max} nm	
Compound	UV spectroscopy	UMSP-80
FAXX	236	238, 242
	300	304
	324	324
PAXX	230	234, 236
	294	286
	312	312, 314

Unpublished results by R.D. Hartley.

in cell walls *(13)* has been used to define nonlignified and nonbiodegradable cell walls. The positive histochemical reactions of parenchyma bundle sheath 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 *(14)*.

Further evidence for a prominent role for ester-linked phenolic acids is shown by ultraviolet (UV) absorption microspectrophotometry and biodegradation studies of specific cell walls *(15–17)*. These studies were made possible by the use of UV-generated illumination and a scanning monochromator designed into an optical microscope. Further, the preparation of specific compounds, namely coniferyl lignin *(18)* and isolated ferulic acid ester-linked to arabinose linked to xylose units (FAXX) and similar structures but with *p*-coumaric acids (PAXX) *(19)*, allowed for location of these various aromatic compounds within cell wall types. Specific absorbances for the two ester-linked compounds by the microspectrophotometric system are confirmed by UV spectroscopy (Table 2).

UV absorption studies of cell wall types undertaken with the Carl Zeiss, Inc. (Thronwell, NY) UMSP-80 microspectrophotometry system are shown in Fig. 2. The λ_{max} near 280 nm is typical for lignin (Fig. 2A). The ester-linked phenolic acids show a bathochromic shift from 280 to a shoulder near 290 nm with λ_{max} near 320 nm. FAXX and PAXX can be differentiated by λ_{max}'s near 326 and 314 nm, respectively (Fig. 2B). With absorbances for these compounds and compositional data on aromatic compounds in grasses, information can be 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 result suggests the presence of lignin and phenolic acid esters within these highly lignified (as shown with AP), nondegradable cell walls. Parenchyma bundle sheaths (PBS), which are a prominent part of the Kranz anatomy of warm-season grasses (Table 1) *(20)*, show a UV spectral pattern identical to phenolic acid esters, suggesting little or no polymeric lignin but a prevalence of

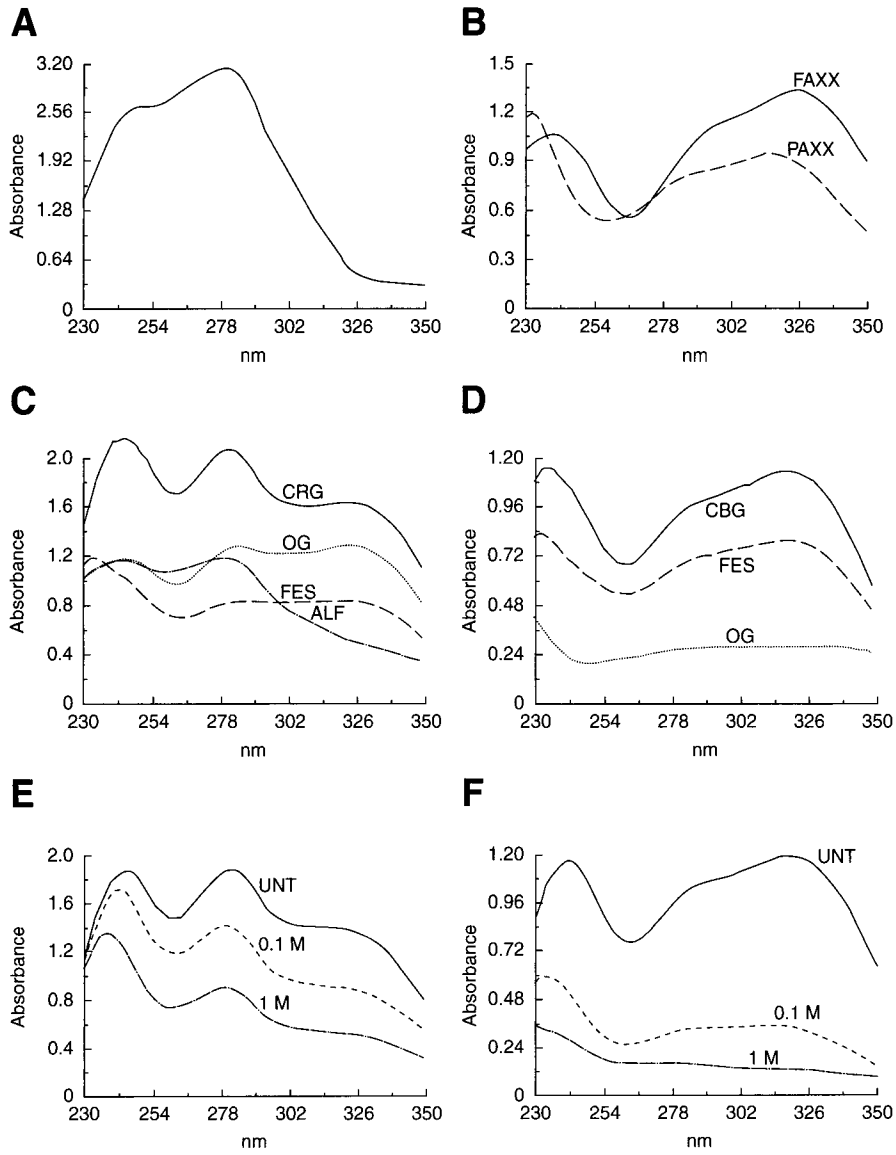

Fig. 2. UV absorption microspectrophotometry. **(A)** Synthesized coniferyl lignin showing λ_{max} at about 280 nm. **(B)** Phenolic acids esters linked to FAXX and PAXX showing a shoulder near 290 nm and λ_{max} at 324 nm for FAXX and 313 for PAXX. **(C)** Highly lignified mestome sheath cell walls of the warm-season Coastal bermudagrass (CBG), cool-season grasses fescue (FES) and orchardgrass (OG), and the legume alfalfa (ALF). Grasses have high-absorbances indicative of lignin and phenolic acids, whereas alfalfa has only the absorbance indicative of lignin. **(D)** PBS of grasses showing strong absorbance indicative of phenolic acid esters in CBG and weaker ones in FES and OG. **(E)** Bermudagrass mestome sheath untreated (UNT) and treated with 0.1 *M* and 1 *M* levels of NaOH. Treatment with NaOH removes absorbance indicative of ester-linked phenolic acids but leaves absorbance indicative of lignin. **(F)** Bermudagrass parenchyma bundle sheath untreated and treated with 0.1 *M* and 1 *M* levels of NaOH showing progressive loss of UV absorbance indicative of ester-linked phenolic acids, with 1 *M* NaOH removing all UV absorbance.

these esters. The slow to partial degradation pattern (Table 1) of warm-season PBS coincides with the presence of phenolic acid esters. In contrast, PBS of cool-season grasses have little to no UV absorbance indicative of lignin or phenolic acids (Fig. 2D), and are rapidly and completely degraded (Table 1). Further evidence for the presence of ester-linked phenolic acids within these tissues is shown in studies using 0.1 *M* and 1 *M* NaOH treatments to compare untreated mestome (Fig. 2E) and PBS (Fig. 2F) cell walls in bermudagrass. The progressive removal of absorbance with increasing NaOH levels suggests alkali-labile, ester linkages. For mestome cell walls, the absorbance near 280 nm is indicative for lignin remains but is reduced. In contrast, the absorbances at 290 and 320 nm for PBS are totally removed.

The use of histochemical stains for lignin, UV absorption microspectrophotometry for lignin and ester-linked phenolic acids, and response of cell types to fiber-degrading, rumen microorganisms provides information on factors influencing recalcitrance of lignocellulose. Certain cell wall types are highly lignified and recalcitrant to biodegradation, even to the potent fiber-degrading, rumen microbial system. However, in warm-season grasses, living, nonlignified tissues are often slow to degrade owing to the presence of ester-linked phenolic acids. The nonlignified cell types in cool-season grasses, in contrast, lack phenolic acid esters and are rapidly degraded (Fig. 2D, Table 1).

Increasing levels of aromatics in cell types, shown by UV absorption, and biodegradability was followed in grass stems *(21)*. In young cell walls, the epidermis, sclerenchyma ring, and vascular tissue gave spectra similar to FAXX and PAXX and were partially degraded, with only the middle lamellae resistant. As the grass internode matured, UV spectral patterns were similar to those for xylem cells with lignin and FAXX/PAXX, with degradation occurring only in cell walls near the cell lumen. In young parenchyma, UV absorbance was low and biodegradability high. As parenchyma cells matured, middle lamellae were the most resistant portions of the cell wall, and UV absorbance spectral patterns resembled those for FAXX/PAXX.

Grassses, especially warm-season species, are high in the phenolic acid–polysaccharide complexes such as FAXX and PAXX and other, related esters *(19)*. The lack of biodegradability is more associated with lignin, but these complexes likely are the predominant factor influencing recalcitrance in living, nonlignified tissues like PBS and epidermis of leaves and mature stem parenchyma. Amounts of these cell wall types are substantial in warm-season grasses.

Biological Strategies to Overcome Recalcitrance

Plant Breeding

The presence of lignin and aromatics within plants is a protective mechanism against pathogens. Plant breeding to remove these aromatics can result in extremely susceptible plants. An extensive breeding program

Table 3
Comparison of Cell Wall Biodegradability and Chemical Composition of Coastal/Coastcross I

	Leaf dry weight loss			Area of PBS	UV absorbance			
	24 h	38 h	72 h	After 24 h	Parenchyma bundle sheath		Mestone sheath	
					λ_{max}	A	λ_{max}	A
Coastal	24%[a]	36%[a]	42%[a]	350 μm²[a]	291	1.2[a]	285	1.8
					318	1.3[a]	318	1.8
Coastcross I	34%[a]	49%[a]	57%[a]	196 μm²[a]	291	0.8[a]	288	2.0
					322	0.9[a]	321	1.9

From ref. *15*.
[a]Different between cultivars ($p \leq 0.05$).

that has existed for over 70 yr in the Agricultural Research Service of the United States Department of Agriculture (ARS-USDA) in Tifton, Georgia, has produced several new varieties of sustainable bermudagrasses with improved biodegradability *(22)*. In many of these varieties, the improved biodegradability is related to lowered levels of aromatics. One notable example is Coastcross I bermudagrass (CC I) *(23)*. We studied CC I and one of the parents in the cross, namely Coastal bermudagrass (CBG), which is extensively grown as a forage in the southern United States.

In direct comparison of the biodegradation of leaf blades, CC I was significantly higher in rate and extent of biodegradation compared with CBG (Table 3). Transmission electron micrographs of PBS cell walls colonized and degraded by rumen bacteria further provide evidence that these walls in CC I, although similar in structure, are considerably more biodegradable than those of CBG (Fig. 3). UV absorption data provide an explanation for the greater biodegradability of CC I in showing a significantly lower absorbance of FAXX/PAXX type compounds. The mestome sheath cells, recalcitrant to biodegradation in both cultivars, have similar spectral patterns. In terms of cell wall biodegradability, data support the fact that living, nonlignified cell walls have lower levels of phenolic acid esters in CC I, indicating that these compounds limit biodegradation and such plant breeding can be a strategy to improve fermentation.

A substantial part of ARS-USDA's program for bioenergy crops relies on research by plant breeders. Collaborative work is needed where information such as that on CBG and CC I can provide a comprehensive decision on the most appropriate bioenergy crops. For example, the amount and the susceptibility of phenolic acid esters could be factor chosen for improved fermentation and production of high-levels of aromatic coproducts, such as ferulic acid. Indeed, previous work has shown variation in the germplasm of bermudagrasses that might be exploited in these aspects of bioenergy crops *(24)*.

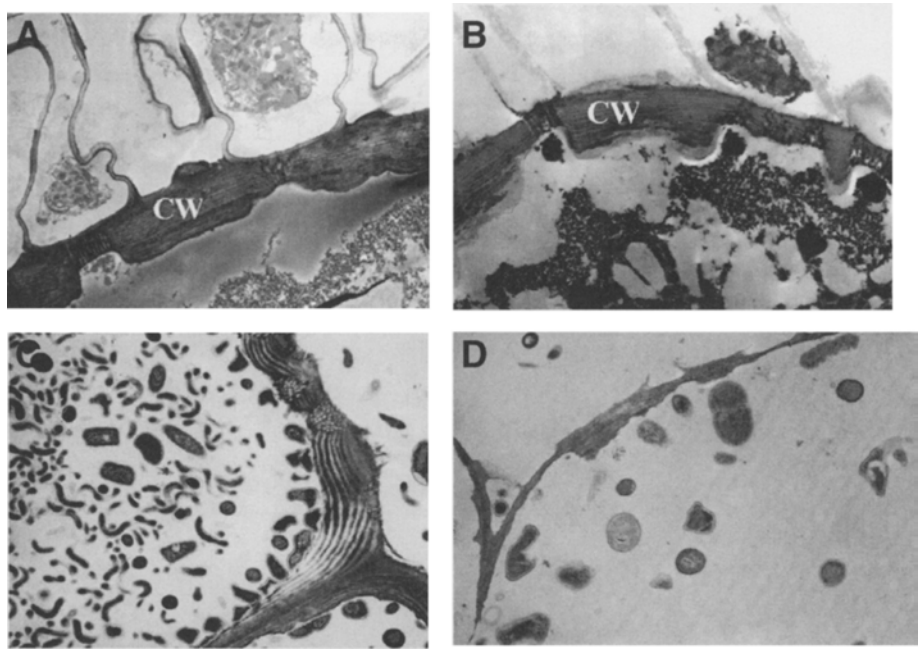

Fig. 3. Comparative biodegradation of parenchyma bundle sheath cells walls (CW) of CBG and CC I undigested and 48 h incubation with the potent fiber-digesting action of rumen microorganisms. **(A)** Cell wall of undigested parenchyma bundle sheath of CBG. **(B)** Cell wall of undigested parenchyma bundle sheath of CC I. **(C)** Attack of parenchyma bundle sheath of CBG by rumen bacteria, with slight pitting at this time. **(D)** The PBS of CC I is more susceptible to bacterial attack, with substantial erosion of the cell wall material.

Lignin-Degrading Microbes and Enzymes

White-rot fungi are recognized as the most active lignin-degrading microorganisms *(25)*. Oxidative enzymes produced by the fungi, along with catalysts, attack aromatics and produce free radicals, which results in degradation of aromatic compounds. Well-publicized oxidative enzymes include: laccases, manganese peroxidase, and lignin peroxidase. At least one of these enzymes, laccase with an activator, is commercially available.

A variety of white-rot fungi occur in nature, and their activities differ. The common pattern of attack on lignocellulose by these fungi is a simultaneous decay of polysaccharides and lignin *(25)*. However, patterns of decay and degrees of delignification vary among species and even strains, and some species selectively delignify plant material leaving an unprotected and available carbohydrate for further use *(26)*. Studies have been undertaken using different white-rot fungal species to improve forage utilization with mixed results *(27,28)*. We evaluated several species of white-rot fungi, emphasizing mutants and species reported to lack cellulase and to selectively attack lignin *(29)*.

Data in Table 4 review results obtained with two white-rot fungi having different capablilities. *Phanerochaete chrysosporium* is a well-studied

Table 4
Influence of Pretreatment With Species of White-Rot Fungi on Bioconversion of Plant Lignocellulose

	Characteristics of pretreated lignocellulose			
	Residual aromatics		Bioconversion potential	
Fungus	1 *M* NaOH (mg/g)	4 *M* NaOH (mg/g)	Dry weight loss (%)	Volatile Fatty Acids (μ moles/mL)
Untreated	13.0	22.9	34.9	47.9
P. chrysosporium K-3[a]	7.1	18.9	46.9	63.8
C. subvermispora 90031-sp.[b]	5.3	17.8	63.9	85.9

From ref. *29*.

[a]*P. chrysosporium* is a well-known and studied white-rot fungus that produces cellulases, hemicellulases, and lignin-degrading enzymes.

[b]*C. subvermispora* is a white-rot fungus that does not produce cellulase.

white-rot fungus that nonselectively attacks cell wall components, i.e., lignin and carbohydrates *(25)*. *Ceriporiopsis subvermispora* had been reported to lack cellulase, produce manganese peroxide and laccase, and to selectively delignify several wood species *(30,31)*. Before our work, this fungus had not been evaluated in the improvement of grass lignocellulose. Our data indicated that *C. subvermispora* was better able to attack the aromatics in bermudagrass and improved the utilization of the residue over that by *P. chrysosporium*. *C. subvermispora* removed ester-linked phenolic acids and lignin moieties from bermudagrass and significantly improved the fermentation of the delignified material (Table 4) *(29)*.

Release of Phenolic Acids by Esterases

The ester-linked *p*-coumaric and ferulic acids previously discussed in grass cell walls, especially warm-seasons grasses such as bermudagrass, not only limit lignocellulosic bioconversion but offer a potential value-added coproduct. Earlier work on anaerobic fungi, some of the most potent fiber-digesting microorganisms in ruminants and herbivorous animals, showed highly active phenolic acid esterases (Table 5) *(32)*. It is believed that these enzymes promoted the ability of the fungi to attack and partially degrade aromatic-containing tissues.

More recent work has shown the value of cell-free ferulic acid esterase along with hemicellulases, in releasing ferulic acid from a variety of plant materials *(33–35)*. For example, recent extraction of defatted jojoba meal with ferulic acid esterase from *Clostridium thermocellum* released ferulic acid from

Table 5
Phenolic Acids Released From Plant Cell Walls

Rumen fungus culture filtrates	*p*-CA[a] (μg/100 mg cell wall)	FA[b] (μg/100 mg cell wall)
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

From ref. *32*.
[a]*p*-Coumaric acid.
[b]Ferulic acid.

Table 6
Effect of Pretreatment With a Commercial Ferulic Acid Esterase[a]

Fraction	Treatment[b]	Dry weight loss (%)	Compounds in filtrates after treatment (mg/g)			
			p-CA[c]	FA[d]	Xylose	Glucose
Corn leaf	Cellulase	41	0.14	0.33	8.0	53.0
	E + C	62	0.32	0.58	36.7	125.0
Corn stem pith	Cellulase	17	0.26	0.22	23.0	68.6
	E + C	29	0.94	0.87	34.0	83.8
Bermuda leaf	Cellulase	16	0.19	0.38	8.0	17.8
	E + C	21	0.61	1.02	30.8	141.3
Corn fiber	Cellulase	27	0.04	0.05	2.6	36.7
	E + C	48	0.23	2.69	8.2	159.3

Adapted in part from ref. *35*.
[a]Depol 740L.
[b]Cellulase incubation for 72 h; E + C incubation with esterase (Depol 740L) for 24 h, removal of filtrate, and subsequent incubation with cellulose 72 h. Values for E + C are summed from the two incubations.
[c]*p*-Coumaric acid.
[d]Ferulic acid.

seven simmondsin ferulates *(34)*. Recently, we evaluated Depol 740 L (Biocatalysts, Ltd., Pontypridd, Wales, UK), a commerical mixture containing ferulic acid esterase, as a pretreatment for grasses before saccharification with cellulases and bioconversion of bermudagrasses *(24)* and corn stover fractions *(35)*. Pretreatment with ferulic acid esterase and saccharification with cellulase is shown for a variety of grass lignocelluloses (Table 6). In all cases, esterase improved release into the filtrate of *p*-coumaric acid, ferulic acid, xylose, and glucose. Particular plants and fractions were more susceptible for release of these particular compounds *(24,35)*. The use of esterases to release ferulic acid