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Enzyme Pretreatment of Grass Lignocellulose for Potential High-Value Co-products and an Improved Fermentable Substrate†

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Introduction

Crops such as switchgrass (*Panicum virgatum* L.), bermudagrass (*Cynodon dactylon* L.), or napiergrass (*Pennisetum purpureum* Schumach.) have the capacity to produce large quantities of lignocellulose for biofuel *(1)*. To facilitate use of lignocellulosic material for ethanol, it will be necessary to determine cost-efficient pretreatments to enhance the conversion to fermentable sugars. The lignified residual products from ethanol production could also provide a value-added co-product for industrial feedstocks (e.g., nutritional antioxidants, ultraviolet absorbers, resins).

Pretreatment of lignocellulose to increase the rate and extent of hydrolysis by chemicals, enzymes, or microorganisms must do the following: (a) improve action of enzymes to produce sugars, (b) avoid loss of potential sugars, (c) avoid formation of inhibitory by-products, (d) be cost effective, and provide a feedstock stream for aromatic co-products *(2)*. Pretreatment of lignocellulose currently consists of mechanical processing, acid hydrolysis, alkali swelling, ammonia, steam and other explosive techniques, and exposure to supercritical fluids *(2–4)*. Mild acid and mild base are proven pretreatments to improve biodegradation of plant materials. Aromatic

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compounds such as lignin or phenolic acid esters are the major unreactive residues in grass lignocellulose *(5)*. Commercial enzymes to remove lignins are not available, except for laccases. However, ferulic acid esterases, which break the ester bond between arabinose and ferulic acid, are available and were used in this study. Exploration of enzymes that separate aromatic constituents from fiber carbohydrates could potentially result in alternative processing methods with potentially attractive features.

Bermudagrass and napiergrass have potential as biomass feedstocks. Napiergrass yields high amounts of dry biomass *(6)*, and is well adapted to the Southern Coastal Plain area. Bermudagrass is already grown on 10–15 million acres throughout the southern United States for hay and forage, and the genetic improvements in digestibility (7–9) could be directly related to the ability to convert the biomass to ethanol. The ferulic acid linkages between lignin and cell wall polysaccharides reduce microbial ability to break down cell walls *(10)* and though most ruminal microorganisms do not possess enzymes to break ether linkages, they do have esterases that can break the ferulate ester linkages *(11)*. These same lignocellulosic linkages can also have a direct effect on polysaccharidases used in a biorefinery setting.

The purpose of this study was to test lignocellulosic enzyme pretreatments on genetically diverse bermudagrass and napiergrass for increased production of fermentable substrates and aromatic co-products from biomass.

Methods

Production, Collection, and Processing of Biomass Material

Bermudagrass and napiergrass plots were fertilized with 227 kg 5:10:15 (N, P_2O_5, K_2O) on March 11, 2003, then staged on April 23, 2003 by mowing bermudagrass plots to 10 cm, napiergrass plots to 20 cm, and applying an additional application of 34 kg of nitrogen. After 4 wk, portions of the plots were mowed to 10 and 20 cm for bermudagrass and napiergrass, respectively, to obtain 4- and 8-wk-old growths. On June 23, 2003 the height of grasses was measured within the plots for each variety and plant age and plots were cut at 10 cm for bermudagrass and 20 cm for napiergrass. Grass plots were allowed to grow and a second harvest of 4- and 8-wk-old material was harvested on August 14, 2003. Two random samples from the cutting of each variety/age plot were gathered and weighed immediately after cutting. The grass samples were separated into leaf and stem fractions and wet weights taken, and the material was then dried in an oven at 40°C. The dry weight samples were weighed, and ground with a Wiley mill using a 1 mm screen (20 mesh). Ground samples were subjected to *in vitro* dry matter digestibility (IVDMD) as described by Tilley and Terry *(12)*, and for enzyme pretreatment. The IVDMD is a measurement of dry matter digestion or degradation by cow mid-gut microbes that contain digestive enzymes.

Enzyme Pretreatments

Whole ground plant material (0.5 g per tube in triplicate) from 4- and 8-wk-old grass samples from the June harvest were incubated with 1.0 g/tube (4393 U/g) of Depol 740 L, which is one of a range of ferulic acid esterase-containing enzymes (Stuart West, personal communication) from Biocatalysts Ltd (Pontypridd, United Kingdom), in 50 m*M* sodium acetate buffer, pH 5.0, at 37° C for 24 h. The esterase-treated material was centrifuged, and the supernatant was removed and frozen for subsequent chemical analysis. The residue was dried, weighed, and then incubated with similarly buffered cellulase (Sigma C-8546) at 400 U/tube for 72 h. The supernatants and residues were treated as before. The values are presented as the sum of compounds released by the esterase and the cellulase incubations.

Monosaccharide Determination

Monosaccharides were measured by adding 0.2 mL of the enzyme supernatant and 0.2 mL of a standard solution of inositol in a 2 mL vial. The solution was freeze dried, and the simple sugars were measured as their silyl ethers by GCL using DMF as the solvent and Sylon BTZ (Supelco, Bellefonte, PA) [*N,O-bis*(trimethylsilyl)acetamide, trimethylsilylimidazole, trimethylchlorosilane, 3:2:3] as the derivatizing reagent.

Phenolic Acid Determination

For chemical analysis, samples were ground in a SPEX 8000 Mixer Mill. Ester-linked and total *p*-coumaric and ferulic acids were determined by treatment of the ground material with 2 *M* NaOH at room temperature for 24 h. The samples were taken to pH 2.5 with 2 *N* HCl and extracted with ether. After evaporation of the solvent, phenolic acids were measured by GLC as their silyl ethers using *N,O-bis* (trimethylsilyl) trifloroacetamide (BSTFA) as previously described *(13)*.

Fermentations

Fermentations were performed (triplicates) in shake flasks with the ethanol-producing recombinant bacterium *Escherichia coli* strain LY01 *(14)* and supernatants from enzyme pretreatments of the various grasses. Flasks were inoculated at an OD_{550} of 0.1 and incubated at 30°C with shaking (100 rpm) for 24 h. The pH of the medium was 6.5. Growth of the bacterium in enzyme digested grass supernatants was compared to growth in media alone (Luria Bertani [LB] medium containing per liter: 10 g typtone, 5 g yeast extract, 5 g NaCl), and to LB plus added enzymes, buffer, and carbohydrates to assess toxicity due to phenolic acids released from the grasses. The proprietary enzyme mixture of Depol740 contains potentially fermentable carbon sources as stabilizers (Gregg Williams,

Table 1
Mean Plant Height, Leaf/Stem Ratios, Percentage Dry Matter and *In Vitro* Dry Matter Digestibilities (IVDMD) of Bermudagrass (B) and Napiergrass (N) Samples at 4 and 8 wk of Growth

Genotype	Age (wk)	Plant height (cm)	Leaf/Stem	Percentage dry matter	IVDMD–leaf[a]	IVDMD–stem[a]
Coastal (B)	4	36	1.34 ± 0.1	40.34 ± 3.6	47.65 ± 2.3	46.03 ± 0.2
Coastal (B)	8	44	0.94 ± 0.2	42.72 ± 2.7	45.73 ± 3.5	49.66 ± 0.6
Tifton 85 (B)	4	30	1.55 ± 0.4	32.87 ± 1.0	60.13 ± 2.3	58.12 ± 0.9
Tifton 85 (B)	8	54	1.12 ± 0.0	36.69 ± 1.4	56.94 ± 3.0	53.11 ± 2.1
Tifton 44 (B)	4	28	1.46 ± 0.5	38.57 ± 4.2	48.18 ± 3.2	48.04 ± 3.3
Tifton 44 (B)	8	36	1.18 ± 0.4	41.96 ± 3.1	47.99 ± 3.1	42.01 ± 3.5
CC II (B)	4	38	1.90 ± 0.4	33.92 ± 0.8	58.21 ± 0.3	56.65 ± 3.2
CC II (B)	8	55	1.18 ± 0.0	38.67 ± 3.2	51.69 ± 0.4	44.84 ± 2.2
Merkeron (N)	4	100	7.02 ± 1.2	9.25 ± 1.3	68.11 ± 2.2	64.96 ± 3.1
Merkeron (N)	8	210	0.77 ± 0.3	19.87 ± 3.4	62.66 ± 1.6	56.08 ± 1.5

[a]*In vitro* dry matter digestibility (IVDMD) in percentage dry matter disappearance after treatment.

Biocatalyst, pers,. comm.). Ethanol concentrations for the various grass supernatant fermentations were determined by gas chromatography essentially as previously described using a DW 640 capillary column at 60°C and a Shimadzu gas chromatograph *(15)*.

Results

There was considerable variability between species, among bermudagrass varieties, and between ages for percentage dry matter, leaf:stem ratios, and IVDMD (Table 1). The proportion of stem increased over time for all the grasses. Napiergrass (Merkeron) is a bunchgrass that grows extremely tall and develops a stiff stalk over time that constitutes two-thirds of the dry matter weight. The digestibility of the stalk was high, however, in comparison with that of bermudagrass at the same age. Among bermudagrass varieties, Tifton 85 and Coastcross II (CC II) had much higher digestibilities than Coastal and Tifton 44, which is consistent with past results (unpublished data).

Whole plant samples of Tifton 85 were used to test enzyme pretreatments. The esterase treatment released a substantial amount of phenolic acid and sugars in the supernatant, compared to buffer only controls, and an additional amount was released with the cellulase treatment (Table 2). Rhamnose levels were minimal and not included. This procedure was then used to rank the other grass varieties.

Esterase/cellulase pretreatment for genotypes and ages produced trends similar to digestibilities (Table 3). Napiergrass, followed by the

Table 2
Phenolic-Acids and Sugars Released from Tifton 85 Bermudagrass After: Buffer Only, Ferulic Acid Esterase and Cellulase

		Phenolic acids (mg/g)		Sugars (mg/g)				
Age	Treatment	*p*CA	FA	Ara	Xyl	Man	Gal	Glu
4 wk	Buffer[1]	0.14a	0.23a	0.9a	1.1a	0.8a	0.3ab	2.6a
	Esterase[2]	0.62c	0.88c	2.2b	5.7b	2.9b	0.6b	73.1b
	+Cellulase[3]	0.43b	0.40b	1.9a	7.9c	0.4a	0.0a	25.1ab
	E + C[4]	1.05	1.28	4.1	13.7	3.4	0.6	98.2
8 wk	Buffer[1]	0.15a	0.29ab	1.1a	0.7a	0.6a	0.2	0.2a
	Esterase[2]	0.43c	0.55b	2.3a	5.8b	3.9b	0.3	70.7c
	+Cellulase[3]	0.33b	0.20a	1.3a	6.6c	0.0a	0.0	42.3b
	E + C[4]	0.76	0.74	3.6	12.4	3.9	0.3	113.1

[1]Sodium acetate buffer (pH 5.5) for 72 h.
[2]Ferulic acid esterase (Depol L 740, Biocatalysts Ltd., Pontypridd, UK) for 24 h.
[3]Residue from [2] above incubated in cellulase (Sigma C-8546) for 72 h.
[4]Values are those summed from [2] and [3] to give total compounds released.
Letters within columns within ages with different letters differ at $P < 0.05$ using one-way analysis of variance (ANOVA).
*p*CA = *para*-coumaric acid; FA = ferulic acid; Ara = arabinose; Xyl = xylose; Man = mannose; Gal = galactose; Glu = glucose.

Table 3
Percentage Dry Weight (DW) Loss, Ferulic Acid and Free Sugars Released in Filtrate after Pretreatments with Commercial Esterase and Cellulase for Bermudagrass (B) and Napiergrass (N) Genotypes at 4 and 8 wk of Age[a]

Genotype	Age[1] (wk)	Percentage DW loss	Ferulic acid (mg/g)	Arabinose (mg/g)	Xylose (mg/g)	Glucose (mg/g)
Coastal (B)	4	38.5 ± 0.4	1.1 ± 0.3	3.4 ± 0.2	8.8 ± 0.7	87.1 ± 6.7
Coastal (B)	8	40.9 ± 0.2	0.9 ± 0.2	3.6 ± 0.5	7.5 ± 3.0	107.4 ± 4.2
Tifton 85 (B)	4	49.7 ± 0.5	1.3 ± 0.0	4.1 ± 0.3	13.7 ± 0.5	84.0 ± 13.3
Tifton 85 (B)	8	42.2 ± 0.6	0.8 ± 0.2	3.6 ± 0.3	12.4 ± 0.2	113.1 ± 2.7
Tifton 44 (B)	4	38.4 ± 0.4	0.8 ± 0.3	3.3 ± 0.3	9.8 ± 0.7	87.8 ± 1.8
Tifton 44 (B)	8	34.3 ± 0.4	0.8 ± 0.1	3.4 ± 0.9	9.5 ± 1.0	80.3 ± 0.9
CC II (B)	4	45.5 ± 0.9	0.2 ± 0.1	4.8 ± 0.9	14.7 ± 1.8	111.7 ± 9.6
CC II (B)	8	36.4 ± 0.0	1.0 ± 0.1	4.2 ± 0.2	12.5 ± 0.3	116.7 ± 2.4
Merkeron (N)	4	64.4 ± 1.0	0.1 ± 0.0	5.1 ± 1.3	19.7 ± 1.6	125.6 ± 6.5
Merkeron (N)	8	46.4 ± 0.9	0.4 ± 0.0	2.9 ± 0.8	15.9 ± 0.2	95.1 ± 1.1

[1]Plant age in weeks of regrowth.
[a] Values are the sum of subsequent incubations with esterase for 24 h and then cellulase for 72 h.

most digestible bermudagrasses (Tifton 85 and CC II), had the greatest dry weight (DW) loss, and 8-wk-old material was more resistant to dry matter decomposition, except for Coastal. The amount of free sugars increased with 8-wk samples except for genotypes with greater stem percentage (Tifton 44 and Merkeron napiergrass).

Table 4
Ethanol Production (g/L) and Bacterial Growth (Optical Density at 550 nm) of Enzyme-treated 4-wk-old Grass Supernatants After 24 h at 30°C using *E. coli* Strain LY01

	Ethanol (g/L)[a] Supernatants from		Optical density[b] Supernatants from	
Cultivar	Esterase	Cellulase	Buffer + esterase	Buffer + cellulase
Coastal	2.67 ± 0.11	0.94 ± 0.05	2.60 ± 0.3	1.60 ± 0.3
Tifton 85	2.87 ± 0.10	0.92 ± 0.04	2.80 ± 0.2	2.25 ± 0.15
Coastcross II	2.81 ± 0.20	1.06 ± 0.05	2.60 ± 0.1	1.90 ± 0.2
Tifton 44	2.73 ± 0.06	0.73 ± 0.02	2.60 ± 0.1	1.60 ± 0.3
Napiergrass	2.10 ± 0.12	1.03 ± 0.05	1.90 ± 0.2	2.10 ± 0.05
Enzyme, Buffer, LB, + carbohydrate	3.12 ± 0.20[c]	0.50 ± 0.15[d]	3.25 ± 0.25[c]	1.50 ± 0.05[d]

All fermentations received supplemental nutrients in the form of Luria Bertani (LB) broth.
[a]Ethanol production from LB medium alone was 0.59 ± 0.1 g/L.
[b]Optical density from LB medium alone was 1.2 ± 0.2.
[c,d]Carbohydrates were added to LB, buffer, and enzyme mixtures to determine the maximum amount of ethanol production with no phenolics present. ([c]contains LB, esterase enzyme with stabilizers, sodium acetate buffer, and 100 mg glucose, xylose, arabinose, mixture-3:1:1 ratio of sugars per g grass; [d]contains LB, cellulase mixture, sodium acetate buffer, and 29 mg glucose per g grass).

Results from fermentation of the supernatants after the pretreatments indicate that an inhibitory compound (or compounds) is present in the esterase-treated supernatant, as none of the samples reached an optical density as high as the buffer and enzyme control alone (Table 4). Tifton 85 showed about 14% inhibition of growth, while Coastcross II, Coastal, and Tifton all showed approximately 20% inhibition of growth. Growth of *E. coli* strain LY01 was 42% inhibited in esterase supernatants of napiergrass. When the esterase supernatant was removed and the residue was treated with cellulase, all grasses supported growth equal to or greater than that obtained for the control with buffer and enzyme only. Tifton 85 and napiergrass exhibited the highest optical densities under these conditions. The lower optical densities for the cellulase-treated samples overall is presumably due to the low concentrations of sugars in these samples. Most of the sugars and phenolic compounds were liberated during the esterase treatment (Table 2); therefore, more ethanol is produced during fermentation with the esterase-treated samples than with the cellulase-treated samples. Carbohydrates are routinely used in commercially available enzyme mixtures and may explain the higher ethanol concentration in the enzyme, buffer, LB, + carbohydrate samples (LB and buffer alone produce only about 0.59 g ethanol/L). Coastcross II and Tifton 85 exhibited the highest ethanol yields of the grass samples, with the

enzyme, buffer, LB, + carbohydrate mixture reaching higher ethanol concentrations than any other grass supernatants.

Discussion

Tifton 85 Bermudagrass has been established and grown by cattlemen due to high production, high digestibility, and high daily weight gain for grazed cattle compared to Coastal and Tifton 44. Coastcross II has not yet been released but also appears to be a high yielding, highly digestible alternative for growers in the future. Napiergrass has also been a useful forage in many countries. These grasses appear to be candidates as feedstocks for bioethanol production.

The enzyme pretreatment released a larger amount of free sugars from the lignocellulose than buffer alone (Table 2). The actual sugar concentrations could be underestimated due to consumption by contaminating microorganisms as grasses were not autoclaved, nor was a microbial inhibitor added to the enzyme pretreatments in this current study. Sterilization using autoclaving helps to degrade the plant material, and the current study was designed to determine the digestibility of grasses using enzyme treatment alone. A microbial inhibitor was not added in the current study as the material was fermented directly after enzyme treatment. Future studies will compare enzyme digestibility of autoclaved grasses compared with enzyme digestibility studies on non-autoclaved grasses with a microbial inhibitor (such as sodium azide) present to more precisely determine the maximum amount of carbohydrates and phenolic compounds liberated during enzyme pretreatment. Our study suggests the presence of inhibitors in esterase-treated samples inhibited bacterial growth and ethanol production. Esterase-released compounds other than ferulic acid appeared to reduce bacterial growth and ethanol production in napiergrass (Merkeron) more than the phenolics identified from bermudagrass (Table 4). *Escherichia coli* strain LY01 was chosen as the biocatalyst because growth of LY01 *(16)* is only 50% inhibited at ferulic acid concentrations of 0.7 g/L, compared to 100% inhibition of the commonly used ethanol-producing *Saccharomyces cerivisiae* at 0.19 g/L *(17)*. Removing phenolic compounds before fermenting the remaining material will increase overall ethanol yields and could provide a valuable co-product for our process.

In addition to the determination of factors responsible for inhibition of fermentation enzymes, further research will be performed to define genetic differences between perennial grass species and genotypes that exhibit varying levels of phenolic acid and free sugar releases, and to test other pretreatment procedures including hemicellulases. The long-term goal is to determine the more efficient grass feedstock/enzyme pretreatment/recombinant fermentation bacteria combinations for the production of ethanol and phenolic acid co-products. The preliminary data would suggest that napiergrass, Coastcross II, and Tifton 85 bermudagrasses may

be the preferable genotypes to undergo further testing as potential feedstocks for biomass-derived ethanol in the southeastern United States. The greater biomass yield and extensive degradability of napiergrass to sugars for fermentation are cause for further work with this species to improve ethanol production and search for valuable co-products; however, inhibition by esterase treatments needs to be addressed. Tifton 85 bermudagrass has high yields, is extensively established in the southern United States, has high digestibility, and has relatively high levels of releasable phenolic acids that could be exploited for co-products.

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