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Assessment of Bermudagrass and Bunch Grasses as Feedstock for Conversion to Ethanol

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Abstract Research is needed to allow more efficient processing of lignocellulose from abundant plant biomass resources for production to fuel ethanol at lower costs. Potential dedicated feedstock species vary in degrees of recalcitrance to ethanol processing. The standard dilute acid hydrolysis pretreatment followed by simultaneous sacharification and fermentation (SSF) was performed on leaf and stem material from three grasses: giant reed (*Arundo donax* L.), napiergrass (*Pennisetum purpureum* Schumach.), and bermudagrass (*Cynodon* spp). In a separate study, napiergrass, and bermudagrass whole samples were pretreated with esterase and cellulose before fermentation. Conversion via SSF was greatest with two bermudagrass cultivars (140 and 122 mg g^{-1} of biomass) followed by leaves of two napiergrass genotypes (107 and 97 mg g^{-1}) and two giant reed clones (109 and 85 mg g^{-1}). Variability existed among bermudagrass cultivars for conversion to ethanol after esterase and cellulase treatments, with Tifton 85 (289 mg g) and Coastcross II (284 mg g^{-1}) being superior to Coastal (247 mg g^{-1}) and Tifton 44 (245 mg g^{-1}). Results suggest that ethanol yields vary significantly for feedstocks by species and within species and that genetic breeding for improved feedstocks should be possible.

Keywords Biomass · Bioethanol · Bermudagrass · Energy crops

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Introduction

Among the perennial grass species that have been cited as potential feedstocks for production in the Southeast are giant reed (*Arundo donax* L.), napiergrass (*Pennisetum purpureum* Schumach.) and bermudagrass (*Cynodon* spp), which have all shown superior dry matter yields compared to switchgrass. Each has potential production advantages and disadvantages for the Southeast.

In Southeastern United States, a significant portion of arable land is planted in pasture grasses with the most widely grown being bermudagrass. In addition to being popular as a forage crop, bermudagrass has the benefit of having preexisting cultivars specifically bred for increased rumen digestibility. Work on forage rumen digestibility has suggested that the binding of aromatic components to cell wall carbohydrates inhibits enzymatic release of sugars and are found within the more recalcitrant tissues of plants [1]. Lignocelluloses vary in the amount and type of aromatics responsible for recalcitrance; some materials are virtually nonconvertible, i.e., highly lignified, while others are only esterified with phenolic acids and can be modified to provide available carbohydrates [2]. Phenolic acids that occur within grass cell walls (*p*-coumaric and ferulic acids [2]) are associated with lignin, and because they are recalcitrant to biodegradation [3, 4], they serve as a barrier for releasing sugars for subsequent ethanol fermentation [5].

In some cultivars of bermudagrass bred for high digestibility (e.g., Coastcross-1), the level of ester-linked phenolics have been found to be reduced within specific cell wall tissues compared to the parents [6]. Prior studies indicate a negative relationship between both ester- and ether-linked ferulic acid concentrations and extent of digestibility among bermudagrass cultivars [7]. The ferulic acid linkages between lignin and cell wall polysaccharides impede microbial break down of cell walls [8]. Alternatively, in highly digestible bermudagrass Tifton 85, the ratio of ether- to ester-linked phenolic acids has been lowered, resulting in improved bioconversion [9, 10]. Ruminal bacteria and fungi produce enzymes that can break the ferulate ester, but none are able to break the tougher ether linkage. It would be of interest to discover if these same ligno-cellulosic linkages also have a direct effect on enzymatic conversion of biomass to sugars in a biorefinery setting.

Napiergrass has value as feedstock for biomass in Southern United States because of high dry matter yields. In a test at Tifton, Georgia, napiergrass (var. Merkeron) (27,764 kg ha^{-1}) out-yielded Tifton 85 bermudagrass (17,578 kg ha^{-1}) and Alamo switchgrass (16,220 kg ha^{-1}) [11]. Yields of napiergrass lines tested in southern and central Florida, grown on a range of soil and cultural practices including sewage effluent and phosphate mining sites, were between 30,000 and 60,000 kg ha^{-1} $year^{-1}$ [12]. Napiergrass yields in northern areas of the South have ranged from the 20,000 to 30,000 kg ha^{-1} $year^{-1}$ [13]. Other data also supports the observation that napiergrass produces more dry matter than other grasses or legumes [14]. It grows in bamboo-like clumps and may reach 7 m in height. The species is well adapted to soil conditions ranging from low fertility acid soils to slightly alkaline and has good drought tolerance due to its deep fibrous root system [15]. Photosynthetic efficiency and water use efficiency of napiergrass is higher than other crops, including giant reed. These traits could lead to much higher sustainable yields than already attained, reducing acreage needed for biomass feedstocks and reducing transport costs. Giant reed has also been identified as a prime biomass source for fuel and an alternative crop for paper/pulp or wood substitutes. The high yield potential and low input demands of giant reed make it an attractive biomass crop [16].

Little is known on the comparative conversion efficiency of these feedstocks to ethanol via saccharification and fermentation. The objectives of this study were to: (1) compare leaf

and stem material from the three grasses for ethanol production via simultaneous saccharification and fermentation (SSF), and (2) better elucidate the differences between bermudagrass genotypes and napiergrass when fermented with pretreatment enzymes.

Methods and Materials

Study 1: Three Species Comparison

Plant Material Preparation

Mature plant samples of three potential dedicated bioenergy feedstock crops were harvested for evaluation of cell wall characteristics. Three stem samples each of clonal collections from Cicily and Fitzgerald, GA of giant reed (*Arundo donax* L.) and genotypes Merkeron and N190 of napiergrass (*Pennisetum purpureum* Schumach.) were harvested from nursery plots grown at Tifton, GA. on November 1, 2004 after a full season of growth. Samples were cut with a knife at 20 cm from ground level. Three samples each of Coastal and Tifton 85 bermudagrass were harvested by hand scissors on November 1, 2004 from nursery plots that had been staged by cutting to 10 cm on August 9, 2004. Leaves were separated from stems for all samples, and weighed. Samples were then dried, weighed, and ground with a Wiley mill and filtered through a 1-mm screen before analyses.

Digestibility and Fiber Analyses

Ground leaf and stem samples of bermudagrass, napiergrass, and giant reed were subjected to *in vitro* dry matter digestibility (IVDMD) as described by Tilley and Terry [17]. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined sequentially [18] using the Ankom filter bag (Ankom Technology Corp., Fairport, NY) method [19] and sulfuric acid.

Saccharification and Fermentation

Each leaf and stem sample was pretreated and converted to ethanol by SSF in triplicate. Dry weights were determined by drying at 105°C. Samples (1.5 g, dry basis) were mixed in 25 ml Corning bottles with 1.75% *w*/*v* sulfuric acid (8.5 ml) and treated at 121°C for 1 h. Bottles were then cooled to room temperature and neutralized by adding 1.2 ml sterile 10% *w*/*v* $Ca(OH)_2$ solution—$Ca(OH)_2$ was kept in suspension during additions by stirring- and 0.55 sodium citrate buffer (1 M, pH 4.5). Further nutrients were supplied by adding 1.1 ml 10× yeast–peptone (200 g/l peptone, 100 g/l yeast extract). Enzyme loadings consisted of 5 FPU GC 220 cellulase/g biomass, and 12 U Novozyme 188 cellobiase/g biomass. The bottles were finally inoculated with *Saccharomyces cerevisiae* D5A. The inoculum was prepared by transferring the yeast from a glycerol culture stored at −80°C to YPD plates (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose, and 20 g/l agar to solidify), then transferring it to 10 ml YPD at 3°C. It was transferred 18 h later to 25 ml YPD supplemented with 50 g/l glucose at 35°C and allowed to grow for an additional 18 h before being concentrated to an optical density (OD) A600nm=50 in 1× diluent (8.5 g NaCl, 0.3 g anhydrous KH_2PO_4, 0.6 g anhydrous Na_2HPO_4, 0.4 g peptone/l). The yeast was added in the fermentation culture to a final optical density (600 nm, OD) of 0.5,

approximately 0.11 ml/bottle. Bottles were incubated at 35°C with 150 rpm mixing. Bottles were fitted with septa-lined caps and vented with inserted needles for CO_2 exhaust. Fermentations were sampled after 72 h for ethanol and remaining sugars, which were measured by high performance liquid chromatography (HPLC). Samples were analyzed for sugars and acids using a SpectraSYSTEM liquid chromatography system (Thermo Finnigan, San Jose, CA) equipped with an organic acid column (Aminex HPX-87H Column, 300×7.8 mm, Bio-Rad Laboratories, Inc, Hercules, CA) and a refractive index detector (RI-150, Thermo Finnigan).

Study 2: Bermudagrass and Napiergrass Comparison

Plant Material Preparation

Bermudagrass (var. Tifton 85, Tifton 44, Coastal and Coastcross II) and napiergrass (var. Merkeron) plots were fertilized with 225 kg ha^{-1} 5:10:15 (N, P_2O_5, K_2O) on March 10, 2004, then staged on July 20, 2006 by mowing bermudagrass plots to 10 cm napiergrass plots to 20 cm. After 4 weeks, bermudagrass plots were mowed to 10 cm to obtain 4-week old samples. On September 14, 2004 the plots were cut at 10 cm for bermudagrass and 20 cm for napiergrass. Two random samples of cut grass from each variety/age plot were gathered and weighed immediately after cutting. The grass samples were weighed wet before drying in an oven set at 40°C. The dry samples were weighed and ground with a Wiley mill using a 1-mm screen (20 mesh). Ground samples were subjected to enzyme pretreatment.

Whole ground plant material (0.5 g dry weight per tube in triplicate) from 4-week-old bermudagrass and 8-week-old napiergrass samples were incubated with 1.0 g/tube (4,393 IU/g) of Depol 740 l in buffer essentially as previously described [5]. The esterase-treated material was centrifuged, and the supernatant removed and frozen for subsequent chemical analysis. The residue was dried, weighed, and then incubated with similarly buffered cellulase (Sigma C-8546) at 400 IU/tube for 72 h. Samples were stored at −80°C until use in fermentations.

Fermentation Protocol

The inoculum was prepared by transferring *Escherichia coli* LY01 [20, 21] from a glycerol culture stored at −80 °C to Luria Bertani (LB) plates (Fisher Scientific, Fair Lawn, New Jersey) with an additional 20 g/l glucose and 40 mg/l chloramphenicol. Plates were incubated at 35 °C for 18 h. A single colony was transferred to 50 ml LB supplemented with 50 g/l glucose and 40 mg/l chloramphenicol at 35 °C and incubated for 18 h. Bacteria were added in the fermentation culture to a final optical density (550 nm, OD) of 1.0 [22]. To increase sugar concentration for fermentation, the esterase-treated samples were combined with the cellulase-treated samples for fermentations in 125 ml Erlenmeyer flasks with caps. Flasks were autoclaved to reduce potential contamination during fermentation. Filter sterilized Spezyme® CP (4.8 FPU) was added to the fermentations, and flasks were incubated in a shaking water bath (100 rpm) at 35 °C for 24 h. Samples were taken at 0 and 24 h. These were filtered (Spin-X® Centrifuge Tube Filter 0.22 μm) and then analyzed by gas chromatography (Shimadzu GC-8A, Inj/Dec 250 °C, Column 65 °C, 30 m, ID 0.53 mm, Film 3 μm) with 2.0% isopropanol as an internal standard essentially as previously described [22]. Values presented were corrected for ethanol contributions from enzymes containing sugar stabilizers and from media components.

Monosaccharide and Phenolic Acid 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 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 acids were measured by GLC as their silyl ethers using *N*,*O*,bis(trimethylsilyl) trifloroacetamide (BSTFA) as previously described [23].

All data was analyzed statistically using PROC GLM [24] for comparisons among plant material and PROC CORR for correlations among traits.

Results

In vitro dry matter digestibility (IVDMD) of leaves was much higher than for stems except in the case of bermudagrass (Table 1). Neutral detergent fiber (NDF) generally correlated with digestibility as measured by IDVMD. The acid detergent fiber (ADF) of the napiergrass and giant reed leaves and both bermudagrass plant components was significantly different from the woody stem tissue of napiergrass and giant reed. This leaf/stem differentiation was also reflected in results of acid detergent lignin (ADL). In general, ethanol production correlated most closely with ADL ($r=-0.78$, $p<0.0001$), with IVDMD ($r=0.64$, $p=0.0001$), and with ADF ($r=-0.62$, $p=0.0004$).

The most efficient conversion of biomass to ethanol was with leaves and stems of bermudagrass (Table 1). Next, the leaves of the bunch grasses, napiergrass and giant reed, produced the greatest ethanol yields by SSF. Merkeron napiergrass stems also digested well. Tifton 85 plant parts produced significantly more ethanol than Coastal bermudagrass, which is consistent with digestibility data. Substantial sugar remained after 72 h of fermentation (102 g mg^{-1}) for stems of the Cicily giant reed (*Arundo donax*) clone compared to the other samples. The stem of the Fitzgerald clone of giant reed had similar

Table 1 *In vitro* dry matter digestibility (IVDMD), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), and ethanol production of leaf and stem tissues of 12-week-old bermudagrass (*Cynodon* sp.), mature napiergrass (*Pennisetum purpureum*) and giant reed (*Arundo donax*) grown at Tifton, GA. 2004.

Species	Genotype	Tissue	% IVDMD[a]	NDF[a]	ADF[a]	ADL[a]	Ethanol mg/g[a]
Cynodon sp.	Tifton 85	Leaf	47.1 c	77.6 g	35.0 abc	2.93 a	139.6 a
Cynodon sp.	Tifton 85	Stem	49.2 c	77.5 g	37.2 cd	4.04 b	141.1 a
Cynodon sp.	Coastal	Leaf	35.4 e	77.0 fg	33.7 ab	3.85 b	121.7 b
Arundo donax	Cicily	Leaf	54.1 b	67.6 ab	36.7 bcd	3.82 b	109.0 bc
Pennisetum purpureum	Merkeron	Leaf	58.5 a	69.4 bc	36.0 abcd	3.04 a	106.7 bc
Pennisetum purpureum	Merkeron	Stem	43.5 d	74.2 def	48.1 ef	6.95 c	105.3 c
Pennisetum purpureum	N 190	Leaf	46.8 c	73.0 de	38.3 d	3.53 ab	96.7 cd
Arundo donax	Fitzgerald	Leaf	52.4 b	65.5 a	33.7 a	4.14 b	84.8 d
Pennisetum purpureum	N 190	Stem	35.9 e	74.1 def	49.1 f	7.90 d	84.0 d
Arundo donax	Fitzgerald	Stem	22.6 g	75.4 efg	49.9 f	8.98 e	47.2 e
Arundo donax	Cicily	Stem	29.0 f	71.9 cd	45.9 e	8.67 e	44.2 e

[a] Means with the same letter are not significantly different ($p=0.05$).

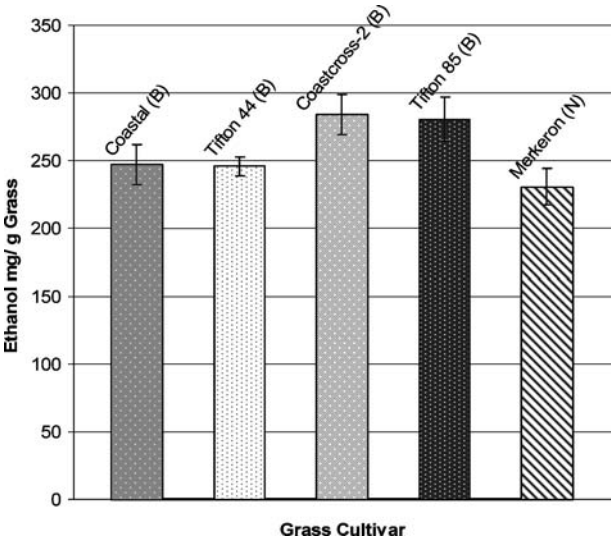

Fig. 1 Ethanol yields (mg/g grass) of 4-week-old bermudagrass cultivars and 8-week-old Merkeron napiergrass harvested at Tifton, GA 2004 and fermented by *Escherichia coli* strain LY01 after pretreatment with esterase and cellulase for 24 h

ethanol yields but much less residual sugar (21.7 mg g^{-1}). Released glucose from leaves of all species was almost completely converted to ethanol as observed by the low residual glucose (2 mg g^{-1}). Inhibitory compounds may be present in giant reed stems, and to a lesser extent, in napiergrass stems. Xylan-associated monosaccharides were equally released during pretreatment of all samples (average of 201 mg g^{-1}), which represents potential increases in ethanol production with xylose-fermenting *Saccharomyces*.

Tifton 85 and Coastcross II yielded the highest amounts of ethanol after enzyme treatment with esterase and cellulase (Fig. 1). These cultivars also had the highest concentrations of glucose after enzymatic pretreatments (Table 2). Pretreatment of Merkeron napiergrass resulted in the greatest dry weight loss. Much of that loss may have been due to hemicellulose as evidenced by the higher yields of xylose, which is the major component of hemicellulose in grasses. Although the Merkeron napiergrass sugar release

Table 2 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 (B) at 4 weeks and napiergrass (N) genotypes at 8 weeks of age[a].

Genotype	Age[b] (weeks)	Percent DW loss (%)	Ferulic acid (mg/g)	P-Coumaric acid (mg/g)	Xylose (mg/g)	Glucose (mg/g)
Coastal (B)	4	33.1 de	0.44±0.04	0.31±0.01	4.7±0.1	84.0±4.7
Tifton 85 (B)	4	41.8 b	0.64±0.02	0.46±0.01	9.2±0.4	112.2±2.0
Tifton 44 (B)	4	32.2 e	0.51±0.11	0.37±0.04	5.5±2.5	78.5±3.8
CC II (B)	4	38.5 c	0.44±0.03	0.30±0.01	6.1±0.1	112.9±4.1
Merkeron (N)	8	55.4 a	0.74±0.10	0.47±0.10	12.7±5.0	91.6±13.8

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

[b] Plant age in weeks of regrowth

was greater than that of Coastal and Tifton 44, the ethanol production was the lowest of the five cultivars tested.

Discussion

The results indicate that bermudagrass would be a superior feedstock for conversion to ethanol via saccharification and fermentation. Under normal harvest procedures, bermudagrass is cut, dried, and baled for hay at maturities of between 4 and 5 weeks. The quality is much better at that time with IVDMD of 60% or better for Tifton 85 [9]. Even at 12 weeks of age and at IVDVD levels of 47%, the observed ethanol yield was much better than observed for napiergrass or giant reed leaves. Superior dry matter yield is not the only aspect to consider when assessing species as potential bioenergy crops in the Southeast. Bermudagrass has the advantage of being an established crop. Growers of bermudagrass hay thus would have an alternative market for hay. If fields cannot be cut in a timely manner for animal forage, older hay would have sufficient quality to be used in an ethanol plant. The ethanol yields from napiergrass and giant reed leaves are comparable to switchgrass (Dien, personal communication); however, stem material is not conducive to fermentation at full maturity when applying a low severity pretreatment (Table 1) and stem made up the majority of the dry matter for giant reed (83%) and napiergrass (59%). The stems would require a harsher pretreatment or may be better suited for thermo-chemical conversion to biofuels. Eight-week-old whole napiergrass (leaves and stems) appears suitable as a feedstock for fermentation (Table 2) under a two or three harvest per year management system.

Bermudagrass yielded more ethanol compared to napiergrass with both the dilute acid pretreatment and enzymatic pretreatments. There appears to be significant enough variation among bermudagrass cultivars (Fig. 1) to warrant breeding and selection for improved cultivars for the biofuels industry. In a previous study, bermudagrasses and napiergrass were treated with esterase alone and the resulting sugars fermented to ethanol. Tifton 85 yielded the most ethanol, followed by Coastcross II, Tifton 44, and Coastal bermudagrass. Ethanol production from napiergrass was lowest of the five grass cultivars tested [5]. The solids were recovered, dried, then treated with cellulase and a second, separate, sugar stream fermented to ethanol. Because of the small volumes of material and the dilute sugar concentrations, the amount of ethanol produced in each individual stream was low. In this study, the grasses were treated with esterase followed by cellulase; however, the samples were combined, and washing steps were reduced in an effort to keep the sugar stream more concentrated. Adding esterases and cellulases together in one pretreatment was not as effective as sequential treatments (data not shown), presumably due to inhibition of the cellulases by the phenolics released by the esterases. The combined sugar streams in buffer were autoclaved to prevent microbial contaminant interference with sugar fermentation to ethanol. Regardless of the differences in protocols, the same hierarchy of performance was observed with Tifton 85 and Coastcross II producing more ethanol than Tifton 44 and Coastal for the bermudagrasses and Merkeron napiergrass producing the least amount of ethanol in both studies. Results from the current study illustrate greater differences in some of the cultivars than observed in the previous study [5]. Phenolic compounds, liberated during the enzyme pretreatment, are known to have an inhibitory effect on microorganisms; however, ferulic acid and para-coumaric acid concentrations alone do not explain the reduction in ethanol yield from Merkeron napiergrass. Future studies will examine this inhibition more closely and will compare fermentations with phenolics removed before inoculation.

Overall ethanol yields were much higher in the second study which used enzymatic pretreatments. Bermudagrass and napiergrass plant samples were much less mature in the second study, but more importantly, fermentation was enhanced by using *Escherichia coli* strain LY01, which converts xylan sugars and the glucans. *Saccharomyces cerevisiae* D5A that was used in the first study does not ferment xylans. Ethanol yields were brought closer to maximization by combining the esterase–cellulase pretreatment of younger plant material and the more efficient fermenting agent.

The significant correlation between IVDMD for forage and ethanol production in these results indicate that breeding for improved forage quality via IVDMD may be sufficient for selection of improved feedstock for ethanol. More work is required to determine whether selecting for lignin content or ADL would be an effective indirect method of measuring for conversion efficiency.

In conclusion, bermudagrass appears to be a viable feedstock for ethanol. Leaves of the bunchgrasses napiergrass and giant reed have potential as feedstock through fermentation; however, due to the high stem to leaf ratio of giant reed, it would be more suited to thermochemical conversion. Sufficient genetic variability among bermudagrass lines should allow for improvement in ethanol yields through breeding.

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