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Biotechnology for Fuels and Chemicals

The Twenty-Ninth Symposium

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Proceedings of the Twenty-Ninth Symposium
on Biotechnology for Fuels and Chemicals
Held April 29–May 2, 2007, in Denver, Colorado

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

William S. Adney

National Renewable Energy Laboratory

Golden, CO 80401-3393

The Twenty-Ninth Symposium on Biotechnology for Fuels and Chemicals was held April 29 - May 2, 2007 in Denver, Colorado. Continuing to foster a highly interdisciplinary focus on bioprocessing, this symposium remains the preeminent forum for bringing together active participants and organizations to exchange technical information and update current trends in the development and application of biotechnology for sustainable production of fuels and chemicals. This annual symposium emphasizes advances in biotechnology to produce high-volume, low-price products from renewable resources, as well as to improve the environment. Topical foci include advanced feedstock production and processing, enzymatic and microbial biocatalysis, bioprocess research and development, opportunities in biorefineries, commercialization of biobased products, as well as other special topics.

Advances in commercialization of bioproducts continued apace this year, and the level of interest and excitement in expanding the use of renewable feedstocks continued to grow. Nonetheless, significant techno-economic challenges must be overcome to achieve widespread commercialization of biotechnological fuels and chemicals production, particularly to move the feedstock base beyond primarily sugar crops and cereal grains (starch) to include holocellulose (cellulose and hemicellulose) from fibrous lignocellulosic plant materials.

Participants from academic, industrial, and government venues gathered to discuss the latest research breakthroughs and results in biotechnology to improve the economics of producing fuels and chemicals. The total of 702 attendees represented an all-time conference high; this is almost a 46% increase over the 2006 conference attendance in Nashville. Of this total, approximately 45% of attendees were from academia (about half of this, 14% of the total attendees, were students), 31% were from industry, and 22% were from government. A total of 78 oral presentations (including Special Topic presentations) and 350 poster presentations were delivered. The high number of poster submissions required splitting the poster session into two evening sessions. (Conference details are posted at http://www.simhq.org/meetings/29symp/index.html).

Almost 40% of the attendees were international, showing the strong and building worldwide interest in this area. Nations represented included Armenia, Australia, Belgium, Brazil, Canada, People's Republic of China, Republic of China, Denmark, Finland, France, Germany, Ghana, Hungary, India, Italy, Japan, Korea, Mexico, New Zealand, Nigeria, Norway, Portugal, South Africa, Spain, Sweden, Thailand, The Netherlands, and United Kingdom, as well as the United States.

One of the focus areas for bioconversion of renewable resources into fuels is conversion of lignocellulose into sugars and the conversion of sugars into fuels and other products. This focus is continuing to expand toward the more encompassing concept of the integrated multiproduct biorefinery—where the production of multiple fuel, chemical, and energy products occurs at one site using a combination of biochemical and thermochemical conversion technologies. The biorefinery concept continues to grow as a unifying framework and vision, and the biorefinery theme featured prominently in many talks and presentations. However, another emerging theme was the importance of examining and optimizing the entire biorefining process rather than just its bioconversion-related elements.

The conference continued to include two Special Topics sessions devoted to discussing areas of particular interest. This year the two topics were international biofuels developments and the evolving attitudes about biomass as a sustainable feedstock for fuels, chemicals and energy production. The first Special Topic session was entitled "International Energy Agency (IEA) Task #39-Liquid Biofuels." This session focused on recent international progress on production of liquid biofuels and was chaired by Jack Saddler of the University of British Columbia. The second Special Topic session was entitled, "'Outside of a Small Circle of Friends': Changing Attitudes about Biomass as a Sustainable Energy Supply," and was chaired by John Sheehan of NREL. This session focused on the evolving perceptions within the agricultural producer and environmental and energy efficiency advocacy communities that biomass has the potential to be a large volume renewable resource for sustainable production of a variety of fuel, chemical, and energy products.

The Charles D. Scott award for Distinguished Contributions in the field of Biotechnology for Fuels and Chemicals was created to honor Symposium founder Dr. Charles D. Scott who chaired this Symposium for its first ten years. This year, the Charles D. Scott award was presented to

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Session 1A: Feedstock Genomics and Development
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Steve Thomas, *Ceres, Inc.*

Session 1B: Microbial Catalysis and Engineering
Chairs: Lisbeth Olsson, *BioCentrum-DTU*,
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Session 2: Enzyme Catalysis and Engineering
Chairs: Sarah Teter, *Novozymes*
Steve Decker, *National Renewable Energy Laboratory*

Session 3: Bioprocess Separations and Process R&D
Chairs: Robert Wooley, *National Renewable Energy Laboratory*
Dhinakar Kompala, University of Colorado

Session 4: Biorefineries and Advanced System Concepts
Chairs: David Glassner, *Natureworks, LLC*
Mark Laser, *Dartmouth College*

Session 5A: Feedstock Preprocessing and Supply Logistics
Chairs: Robert Anex, Iowa State University
Corey Radtke, Idaho National Laboratory

Session 5B: Feedstock Fractionation and Hydrolysis
Chairs: Susan Hennessey, E.I DuPont de Nemours and Co.
Nathan Mosier, Purdue University

Session 6: Industrial Biofuels and Biobased Products
Chairs: Dale Monceaux, AdvanceBio, LLC
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Acknowledgments

The continued success of the Symposium is due to the many participants, organizers, and sponsors, but is also the result of significant contributions by numerous diligent, creative and talented staff. In particular, Jim Duffield of NREL, conference secretary, provided timely advice and heroic persistence while maintaining an unfailingly upbeat attitude.

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28. “Proceedings of the Twenty-Eighth Symposium on Biotechnology for Fuels and Chemicals” (2005), Appl. Biochem. Biotechnol. **121–124**.

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

The Thirtieth Symposium will be May 4-7, 2008 in New Orleans, Louisiana. More information on the 28th and 29th Symposia is available at the following websites: http://www1.eere.energy.gov/biomass/biotech_symposium/ and http://www.simhq.org/meetings/29symp/index.html. We welcome comments or discussions relevant to the format or content of the meeting.

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Introduction to Session 1A: Feedstock Genomics and Development

Wilfred Vermerris

Genomics research aimed at improving bioconversion properties of feedstocks received a major impetus as a result of the Feedstock Genomics program jointly operated by the U.S. Department of Energy (DOE) and the U.S. Department of Agriculture (USDA). In addition, oil company BP established the Energy Biosciences Institute in collaboration with the University of California-Berkeley, Lawrence Berkeley National Laboratory, and the University of Illinois in Urbana-Champaign. This was followed later on in the year by the establishment of three DOE-funded bioenergy centers. The need to switch from petroleum-based duels to biofuels was underscored by the report of Working Group II of the United Nations-sponsored International Panel on Climate Change (IPCC), in which the wide-spread effects of greenhouse gas emissions on the global climate were presented. IPCC and former U.S. vice-president Al Gore received the 2007 Nobel Peace Prize for their efforts to quantify and disseminate the effects of global warming.

The presentations in Session 1A reflected this new impetus, as evidenced by two oral presentations from recipients of USDA–DOE funding, Dr. William Rooney (Texas A&M University, College Station, TX, USA) and Dr. Rick Dixon (Noble Foundation, Ardmore, OK, USA). Dr. Rooney discussed his research on the development of sorghum for bioenergy production. Photoperiod-sensitive sorghums do not transition to the reproductive stage and can produce large amounts of biomass, as high as 27 Mg ha^{-1}. He also discussed genetic approaches to identify genes controlling sugar accumulation, cell wall composition, and biomass production in sorghum. Dr. Dixon presented his research on the transgenic down-regulation of monolignol biosynthetic genes in alfalfa. Conversion of alfalfa biomass appeared to be primarily dependent on lignin content as opposed to lignin subunit composition. The down-regulation of some of the genes resulted in a noticeable reduction in the total amount of biomass, an undesirable side effect. The impact of lignin content and composition was also discussed by Dr. William Anderson (USDA, Tifton, GA, USA), Dr. James Coors (University of Wisconsin-Madison, WI, USA), and Dr. Gautham Sarath (USDA, Lincoln, NE, USA) in their presentations on Bermudagrass, maize, and switchgrass, respectively. In maize, lignin content appeared to impact biomass conversion

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properties, just like in alfalfa, whereas in Bermudagrass and switchgrass lignin subunit composition appeared to be a more critical factor.

The need to establish reliable methods for the evaluation of biomass conversion properties was expressed in several of the presentations. Methods that were originally developed for the analysis of forage quality seem to provide a reasonable approximation of biomass conversion potential in some species (maize), but not in other species (Bermudagrass). Ms. Michelle Serapiglia (SUNY-ESF, Syracuse, NY, USA) discussed how thermogravimetric analyses may be applicable to determine lignin content and composition in shrub willow. The oral session was concluded with a presentation by Dr. Steven Thomas (Ceres, Inc., Thousand Oaks, CA, USA) on ways in which genetic diversity in switchgrass can be catalogued and exploited for the development of superior germplasm.

Several poster presentations in this session focused on the chemical basis of biomass conversion and the development of methods to determine which features contributed to a more rapid bioprocessing. Approaches included the use of atomic force microscopy, fluorescently labeled cellulases, near infrared reflectance spectroscopy and fluorescence spectroscopy. Other topics represented in the poster presentations included the production of cell wall-degrading enzymes *in planta*, and plant breeding approaches, including the incorporation of mutations and the introduction of transgenes to facilitate biomass processing of a variety of species, including sorghum, wheat, corn, shrub willow, and switchgrass.

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High-resolution Thermogravimetric Analysis For Rapid Characterization of Biomass Composition and Selection of Shrub Willow Varieties

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Arthur J. Stipanovic · Lawrence B. Smart

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Abstract The cultivation of shrub willow (*Salix* spp.) bioenergy crops is being commercialized in North America, as it has been in Europe for many years. Considering the high genetic diversity and ease of hybridization, there is great potential for genetic improvement of shrub willow through traditional breeding. The State University of New York—College of Environmental Science and Forestry has an extensive breeding program for the genetic improvement of shrub willow for biomass production and for other environmental applications. Since 1998, breeding efforts have produced more than 200 families resulting in more than 5,000 progeny. The goal for this project was to utilize a rapid, low-cost method for the compositional analysis of willow biomass to aid in the selection of willow clones for improved conversion efficiency. A select group of willow clones was analyzed using high-resolution thermogravimetric analysis (HR-TGA), and significant differences in biomass composition were observed. Differences among and within families produced through controlled pollinations were observed, as well as differences by age at time of sampling. These results suggest that HR-TGA has a great promise as a tool for rapid biomass characterization.

Keywords Cellulose · Hemicellulose · Lignin · *Salix* · Wood composition

Introduction

Reliance on petroleum-based transportation fuels has raised national concern with respect to homeland security, energy independence, depletion of petroleum resources, and impact on

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the environment. The production of biofuels from dedicated energy crops and agricultural crop residues grown sustainably within the USA could help alleviate these problems. Currently, the vast majority of ethanol fuel produced in the USA is made from a single feedstock, corn grain, harvested from an annual crop. Achieving the goal of replacing 30% of the US petroleum consumption with biofuels and bioproducts by 2030 will require the use of perennial crops as well as the current annual crops [1]. As extraction techniques and conversion processes improve and become more cost effective, sustainable perennial woody crops, such as fast-growing willow shrubs, will become the preferred feedstocks. Shrub willow (*Salix* spp.), a high-yielding perennial crop with a short harvest cycle of only 3 to 4 years, is considered a suitable energy crop for much of North America [2, 3] and can be grown on underutilized agricultural land [3, 4]. There are multiple environmental benefits to growing shrub willow and excellent potential for genetic improvement through traditional breeding [5].

Researchers at the State University of New York College of Environmental Science and Forestry (SUNY-ESF) have developed a breeding program for the genetic improvement of shrub willow for increased biomass production [4]. There are more than 300 species of *Salix* worldwide with little domestication and high genetic diversity [6]. Since 1994, SUNY-ESF has collected and planted more than 750 accessions of shrub willow and established the largest willow-breeding program in North America [3, 4]. From these accessions, breeding efforts begun in 1998 have produced more than 5,000 progeny. Between 1998 and 2007, more than 200 families have been generated through controlled pollination. Crosses completed in 1998 and 1999 produced more than 2,000 individuals that have been screened in field trials for high biomass, form, and disease resistance [4, 7]. Selected groups of superior clones from crosses performed in 1998 and 1999 were planted in selection trials in 2001 and 2002, respectively. Growth improvements as high as 40% greater than a reference clone have been observed [4].

If shrub willow is to be used as a feedstock for the production of bioproducts or biofuels, the bioconversion process must become more efficient and cost effective. This can be partially achieved by selecting varieties with biomass composition that is better suited to the conversion process. Composition of the biomass is critical to the efficiency of processing and product yield, whether it is used to produce liquid fuels such as ethanol or polymers such as biodegradable plastics. Lignocellulosic biomass displays considerable recalcitrance to biochemical conversion because of the inaccessibility of its polymer components to enzymatic digestion and the release or production of fermentation inhibitors during pretreatment. If the ratio of hemicellulose, cellulose, and lignin in a woody biomass feedstock was optimized for the specific biochemical conversion method, then expensive and chemically harsh pretreatment methods could be reduced or avoided [8].

The development of a high-throughput process for the analysis of willow biomass will allow for selection of improved varieties with more favorable biomass composition in the willow breeding program. Traditional wet chemistry techniques for the analysis of biomass require strong acids and time-consuming processes resulting in a method whereby only 20 samples per week per person can be analyzed [9]. Current advancements in analytical methods include infrared spectroscopy (Fourier transform infrared [FT-IR] and near-infrared [NIR]) and pyrolysis molecular beam mass spectroscopy (pyMBMS) [10–13]. Multivariate analyses are often used in conjunction with these methods. To increase accuracy and improve throughput, development and further improvement of new analytical methods is required.

This project focuses on the development of high-resolution thermogravimetric analysis (HR-TGA) as a rapid, low-cost method for the analysis of biomass composition of shrub willow. The goal is to provide an alternative method for biomass analysis that is faster and more cost effective than existing techniques with comparable or enhanced accuracy. This method can quantitatively resolve complex mixtures based on the characteristic thermal decomposition temperature of each component. It is well established that the pyrolytic decomposition of woody plant tissues in inert atmospheres occurs at the lowest temperature for hemicellulose (250–300 °C), followed by cellulose (300–350 °C) and lignin (300–500 °C) [14]. HR-TGA has already been applied to the analysis of lignocellulosic material and has shown to be useful in compositional analysis [15, 16]. Our work applies this method in analysis of willow varieties produced in the SUNY-ESF breeding program.

Materials and Methods

Source Material and Tissue Collection

Willow stem biomass samples were collected in January 2006 from two field trials growing at the Tully Genetics Field Station (Tully, NY; Table 1). Individuals sampled from the 2001 selection trial have clone IDs with the designation "98XX," where 98 indicates the year of the cross and XX the number of the family. Clones sampled from the 2002 selection trial were bred in 1999 and have IDs beginning with the designation "99." Samples from the reference clones SV1, SX61, SX64, and SX67 were collected from both selection trials. Samples were collected from three replicate plants for each of the 95 clones (Table 1) as follows: 15-cm sections including bark were cut from the base, middle, and top of one representative canopy stem. These stem sections were dried to a constant weight at 65 °C and then ground in a Wiley mill with a 20-mesh screen. The ground material from the three sections of each stem was pooled and homogenized. Each of the three replicates was analyzed in triplicate, for a total of nine analyses per clone. Samples from the 1999 families

Table 1 Families and reference clones in this study.

Family ID	Species	Number of progeny analyzed
9870	*S. sachalinensis* × *S. miyabeana*	4
9871	*S. sachalinensis* × *S. miyabeana*	4
98101	*S. dasyclados* × *S. miyabeana*	2
9882	*S. purpurea* × *S. purpurea*	4
9970	*S. sachalinensis* × *S. miyabeana*	13
9979	*S. purpurea* × *S. miyabeana*	1
9980	*S. purpurea* × *S. miyabeana*	1
99113	*S. purpurea* × *S. purpurea*	3
99201	*S. viminalis* × *S. miyabeana*	4
99202	*S. viminalis* × *S. miyabeana*	15
99207	*S. viminalis* × *S. miyabeana*	7
99208	*S. viminalis* × *S. miyabeana*	2
99217	*S. purpurea* × *S. miyabeana*	12
99227	*S. purpurea* × *S. purpurea*	2
99232	*S. purpurea* × *S. purpurea*	2
99239	*S. purpurea* × *S. purpurea*	15
SV1	*S. dasyclados*	–
SX61	*S. sachalinensis*	–
SX64	*S. miyabeana*	–
SX67	*S. miyabeana*	–

were collected after the third growing season after coppice, while samples from the 1998 individuals were collected one growing season after coppice. Samples of both ages were collected from the reference clones SV1, SX61, SX64, and SX67.

High-resolution Thermogravimetric Analysis

All willow samples were analyzed using a Thermogravimetric Analyzer 2950 (TA Instruments, New Castle, DE) with the TA Universal Analysis 2000 software. The method used for all samples was "high-resolution dynamic" with a heating rate of 20 °C min^{-1}, a final temperature of 600 °C, a resolution of 4.0, and a sensitivity value of 1.0. The electro-balance was purged with nitrogen at a flow rate of 44 L min^{-1}, and the furnace was purged with compressed air with a flow rate of 66 mL min^{-1}. For each analysis, 10 mg of dry tissue was used.

The percent dry weight for each stem biomass component (hemicellulose, cellulose, and lignin) was calculated by designating weight loss cutoff points on the generated thermogram (Fig. 1). The initial mass of the sample was corrected for water loss (change in weight from starting temperature to around 129 °C). Hemicellulose content was designated to be the weight loss between 245 and 290 °C, cellulose between 290 and 350 °C, and lignin between 350 and 525 °C. These cutoff points were identical for each sample, providing relative differences among the clones.

Statistical Analysis

All statistical analyses were performed using SAS® version 9.1.2 at a critical α=0.05. SAS PROC GLM and PROC NESTED were used to analyze all TGA data and to evaluate the

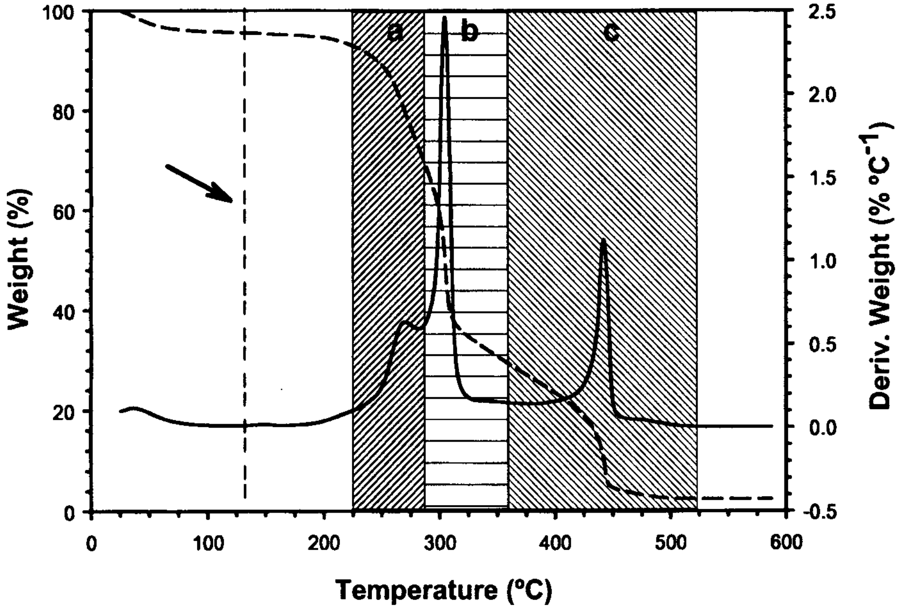


Fig. 1 TGA thermogram of biomass from reference willow clone *S. dasyclados* 'SV1.' *Arrow* indicates cutoff line for water loss correction (129 °C). *Block A*: weight loss representative of hemicellulose (245–290 °C). *Block B*: weight loss representative of cellulose (290–350 °C). *Block C*: weight loss representative of lignin (350–525 °C)

differences in biomass composition. When a significant interaction ($P<0.05$) was observed, Tukey's mean studentized range test was used to determine significant differences among clones. The variance components for the total data set, between and within clones, and within instrumental run were estimated with PROC NESTED. The multivariate analyses PROC CLUSTER and PROC CANDISC (discriminate analysis) were performed to identify groupings among specific clones.

Results and Discussion

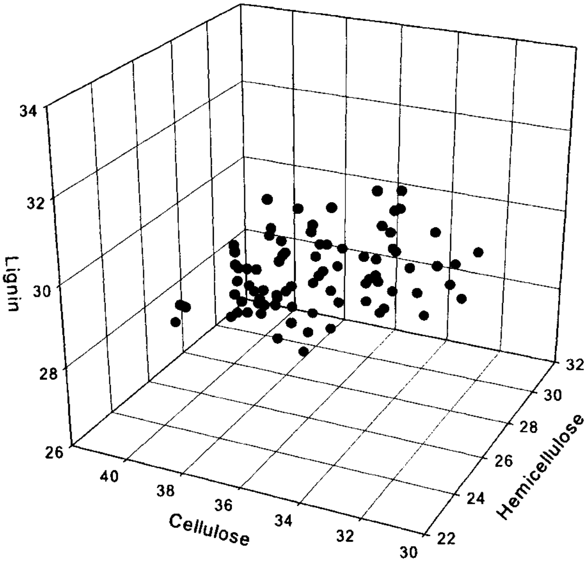
As the breeding and domestication of crops to serve as feedstocks for biofuels and bioenergy is a very recent priority, there is urgent need to focus or refocus the aim of energy crop breeding programs to the optimization of biomass composition, while maintaining and improving high yield as the most critical trait. Characterizing and identifying differences in biomass composition among the varieties produced through conventional breeding demands techniques that are relatively fast, precise, and inexpensive. To refine the selection strategy of the willow breeding program with the aim of identifying varieties that have biomass composition that is well matched with the requirements of the intended downstream conversion technology, we have embarked on the development of HR-TGA as a rapid, low-cost method for analyzing and screening the biomass of hundreds or thousands of unique willow genotypes. Based on the initial results obtained in this study, HR-TGA may be an advantageous tool for the willow breeding program.

Utilizing this HR-TGA method, we were able to identify significant differences in the relative cellulose, hemicellulose, and lignin content among 95 willow clones. Statistical analysis provided variance components among clones, experimental replication, and instrumental replication. The total variation observed in the data set was relatively low, but more than 50% of the total variation was attributed to clonal variation. Instrument variation accounted for a maximum of 25% of total variation. The observed experimental and instrumental variation suggests that either more experimental replications or instrumental runs would help reduce variation, but the error is relatively small compared to the means; therefore, this is not a critical issue. This small error was generated using a remarkably small sample size of only 10 mg, which is indicative of the precision of the instrument. Small sample size, speed of analysis, and the ease of sample preparation for instrumental analysis are other advantages associated with this analytical method. Currently, one instrument can analyze 16 samples per day with a run time per sample of 90 min. As the instrument has an autosampler, it can process 16 samples before more samples need to be loaded. With further refinement of this analysis, the run time might be shortened. In addition, multiple instruments can be utilized to increase the daily throughput.

No discrete groupings or clusters were observed among the clones when plotted in a 3D graph (Fig. 2). Several multivariate analyses were performed, but all proved to be inconclusive and are not presented here. Most of the willow clones analyzed have similar biomass composition; however, there are several clones that have distinctively more or less cellulose, hemicellulose, or lignin (Fig. 2). This could be very important in future selection of willow varieties optimized for a particular application.

Among all clones analyzed, cellulose content ranged from 29 to 40%, hemicellulose content ranged from 23 to 30%, and lignin content ranged from 27 to 35% (data not shown). Individuals with the greatest relative amount of one component were significantly different from individuals with the least amount. The individual willow clones that were selected for analysis were purposefully chosen with an eye to their genetic diversity. In

Fig. 2 3D plot of cellulose, hemicellulose, and lignin components for all the 1999 progeny and reference clones analyzed



building a breeding collection at SUNY-ESF, genetically diverse individuals were collected throughout the mid-western and northeastern USA, in addition to accessions from Japan, China, Ukraine, Sweden, and Canada. The range of cellulose, hemicellulose, and lignin content observed here may be an indication of the genetic diversity present in the various clones and will be very beneficial for future breeding efforts.

In the four largest families of the 1999 progeny, significant differences were observed in cellulose and hemicellulose content among siblings in each family (Table 1; Fig. 3; family 9970 data not shown). Significant differences in lignin composition were observed only in families 99217 and 99239 (Fig. 3). Families 9970, 99202, and 99217 are the result of interspecific hybridization, while family 99239 is the result of an intraspecific cross of *S. purpurea*. The siblings of the intraspecific cross displayed the greatest variability, compared with the siblings of the three interspecific hybrids. Kopp et al. [17] have shown that there can be great variability in seedling height growth among individuals produced from an intraspecfic cross of *S. eriocephala*. The variability among the progeny of intraspecific crosses is interesting in light of genetic studies of *Populus* spp. utilizing extensive amplified fragment length polymorphism analyses that have shown that interspecific variability is significantly greater than intraspecific variability [18, 19].

The willow biomass samples collected 1 year after coppice had significantly greater lignin content and lower cellulose content than the samples collected 3 years after coppice. The mean lignin content for the third-year samples was 29.5%, compared to a mean lignin content of 31.7% for the first-year samples, with the highest mean lignin content for a clone of more than 35% (data not shown). Samples were collected from the reference clones SV1, SX61, SX64, and SX67 after one season and three seasons postcoppice. The differences in composition based on stem age are shown in Fig. 4. Cellulose content was significantly lower in the 1-year-old growth compared to the 3-year-old growth. Inversely, lignin content was significantly higher in the younger growth. Hemicellulose appeared to be unaffected by the difference in years. Lignin content in bark is greater than that of wood [20, 21]; therefore, the greater lignin content in 1-year-old biomass may be due to greater bark content as a result of smaller stem diameters. Analyses with hybrid poplar clones have

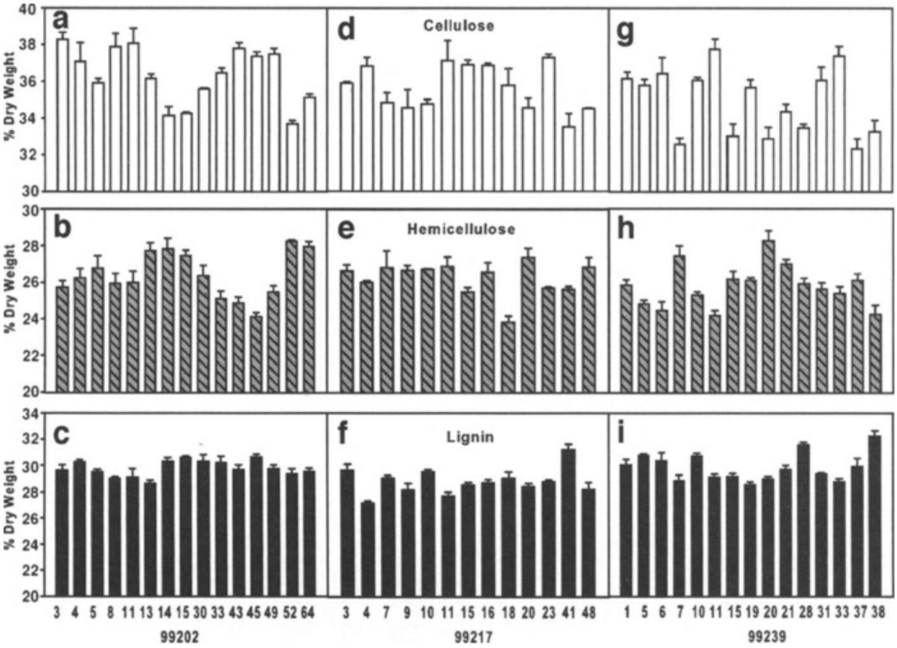


Fig. 3 Cellulose, hemicellulose, and lignin content of progeny in families 99202 (**a–c**), 99217 (**d–f**), and 99239 (**g–i**). *Bars* indicate mean±SE of three experimental replicates, each of which was analyzed using three instrumental replicates. *X*-Axis indicates the clone IDs for specific progeny individuals in each family

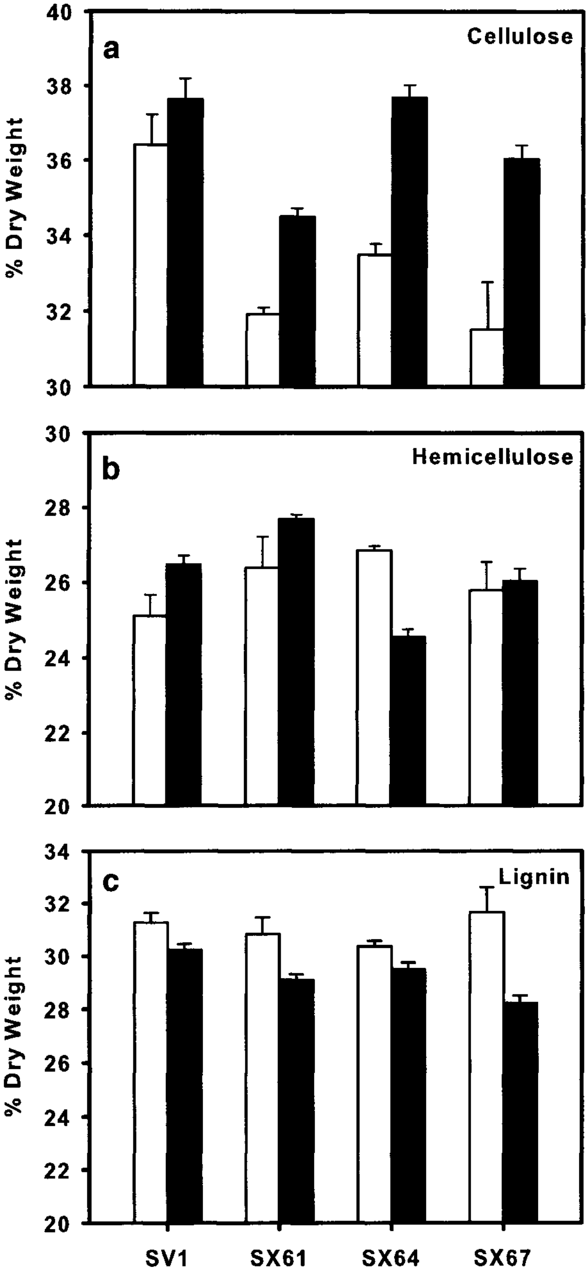
shown that lignin content of bark can be two times greater than that of the wood [20]. In 5-year-old stems from shrub willow stands in Sweden, bark represents approximately 19% of the total biomass. Small-diameter stems had a higher bark-to-wood ratio, and stems larger than 55 mm had a constant bark-to-wood ratio [22]. One-year-old twigs had bark content reaching 54% of the total biomass, compared to 18–27% for older stems [22]. Further analysis of bark content would be required to determine the impact of bark on the overall biomass composition of these clones.

The other analytical methods involving biomass composition that are currently in development (FT-IR, NIR, and pyMBMS) are able to resolve and quantify individual sugar composition. This is not possible with HR-TGA; however, in conjunction with ^{1}H nuclear magnetic resonance (NMR), sugar residues can be identified, and their abundance can be determined. Carbohydrate compositional profiles of lignocellulosic biomass can be accurately quantified based on the 600 MHz ^{1}H-NMR spectrum of unpurified acid hydrolyzates wherein the hemicellulose and cellulose fractions of biomass have been reduced to a mixture of sugars in acidic solution [23].

Conclusions

Preliminary HR-TGA analysis has shown that this technique can be used to identify compositional differences in shrub willow stem biomass among high-yielding clones selected in the breeding program at SUNY-ESF. To further refine this technique, a set of rigorously characterized reference biomass samples of shrub willow clones representing a

Fig. 4 Cellulose (**a**), hemicellulose (**b**), and lignin (**c**) content of different aged biomass samples from the reference clones. *White bars* represent 1 year growth after coppice; *black bars* represent 3-year growth after coppice. *Bars* indicate the mean±SE of three experimental replicates, each of which was analyzed using three instrumental replicates



range of varying compositions are being used to develop a neural network tool that will reliably and accurately interpret HR-TGA thermograms of unknown samples. HR-TGA in combination with ^{1}H-NMR can be a powerful, high-throughput tool used to identify unique compositional features in shrub willow and improve selection in the breeding program.

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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,