0273-2289/1559–0291 (Online)/07/136–140/395–406/$30.00

Ammonia Fiber Expansion Pretreatment and Enzymatic Hydrolysis on Two Different Growth Stages of Reed Canarygrass

Tamika C. Bradshaw, Hasan Alizadeh, Farzaneh Teymouri, Venkatesh Balan, and Bruce E. Dale*

Department of Chemical Engineering and Materials Science, Biomass Conversion Research Laboratory, 2527 Engineering Building, Michigan State University, East Lansing, MI 48824, bdale@egr.msu.edu

Abstract

Plant materials from the vegetative growth stage of reed canarygrass and the seed stage of reed canarygrass are pretreated by ammonia fiber expansion (AFEX) and enzymatically hydrolyzed using 15 filter paper units (FPU) cellulase/g glucan to evaluate glucose and xylose yields. Percent conversions of glucose and xylose, effects of temperature and ammonia loading, and hydrolysis profiles are analyzed to determine the most effective AFEX treatment condition for each of the selected materials. The controls used in this study were untreated samples of each biomass material. All pretreatment conditions tested enhanced enzyme digestibility and improved sugar conversions for reed canarygrass compared with their untreated counterparts. Based on 168 h hydrolysis results using 15 FPU Spezyme CP cellulase/g glucan the most effective AFEX treatment conditions were determined as: vegetative growth stage of reed canarygrass—100°C, 60% moisture content, 1.2 : 1 kg ammonia/kg of dry matter (86% glucose and 78% xylose) and seed stage of reed canarygrass—100°C, 60% moisture content, 0.8 : 1 kg ammonia/kg of dry matter (89% glucose and 81% xylose). Supplementation by commercial Multifect 720 xylanase along with cellulase further increased both glucose and xylose yields by 10–12% at the most effective AFEX conditions.

Index Entries: Ammonia fiber expansion; biomass; enzymatic hydrolysis; pretreatment; reed canary grass; cellulosic ethanol.

Introduction

The United States fuel ethanol industry is currently producing over 4 billion gal/yr of ethanol from starch and sugar sources, primarily corn grain *(1–3)*. However, starches and sugars are only a small fraction of total biomass materials. Biomass is the only potentially renewable source of organic chemicals, organic materials, and liquid transportation fuels *(4)*. Biomass is also relatively inexpensive and compares favorably

*Author to whom all correspondence and reprint requests should be addressed.

with petroleum on a cost per pound basis, and frequently, on a cost per unit of energy *(4)*. However, the cellulosic portion of biomass represents an immense potential source of sugars, which await development of the technology necessary for its economical utilization. Cellulose and hemicellulose form the bulk of most biomass, and effective ethanol production from these components can expand the types and availability of feedstock (5). Cellulosic biomass as an alternate feedstock could provide very large quantities of ethanol with considerable environmental benefits. Bioethanol is an alternative fuel currently used as a gasoline additive to reduce carbon monoxide and other toxic air emissions, ground level ozone formation, and to boost octane.

Unfortunately, there is an as-yet unresolved technical problem impeding large-scale bioethanol production. Lignocellulosic biomass is resistant to enzymatic hydrolysis as a result of many physical and chemical factors. The strong bonding between cellulose, hemicellulose, and lignin within the crystalline regions of lignocellulose makes it rather difficult to degrade the sugar polymers in the cell wall. Enzymatic hydrolysis of this material is possible but requires pretreatment of the cellulosic material before the enzymes can access the sugar polymers *(6)*. Pretreatment is an essential process for enhancing the reactivity toward enzymatic hydrolysis. Pretreatment alters the structure of cellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrates into fermentable sugars *(7)*. The ammonia fiber expansion (AFEX) process might offer both an effective and economically attractive means of increasing yields of fermentable sugars from lignocellulosic biomass *(8)*. AFEX has been shown to decrease cellulose crystallinity and particle size, whereas increasing the surface area exposed to enzymatic attack *(9,10)*.

This article focuses on the effectiveness of AFEX-pretreated reed canarygrass (RCG) at different plant growth stages. RCG is also a potential biofuel raw material, and is a cool season grass alternative to switch grass (SWG). RCG's rhizomatous growth habit also makes it appealing, particularly on soils wherein SWG, a bunchgrass, does not form thick stands and wherein erosion is a problem *(1)*. The vegetative stage (the period when leaves begin to grow) is the earliest stage of grass maturity. It is followed by the jointing, boot, heading, blooming, and finally, the seed development stages.

The objectives of this study were to evaluate suitable AFEX treatment conditions for different growth stages of RCG, estimate glucose and xylose yields obtainable, and to explore the effects of maturity in plants. The most effective AFEX treatment condition that provides the highest percentage conversions for both glucose and xylose were observed for the selected materials at a fixed enzyme loading. An additional objective was to determine the effects of supplementing the cellulase mixture, which is deficient in xylanase (containing approx 1% xylanase), with additional commercial xylanase.

Materials and Methods

Biomass

Materials utilized were the vegetative growth stage of RCG (VRCG) and the seed stage of RCG (SRCG). RCG was dried and ground through a 1-mm screen in a Wiley mill at the Dairy Forage Research Center (Madison, WI). The cell wall carbohydrates (cellulose, xylan, arabinan, galactan, and mannan), soluble carbohydrate (glucose, fructose, sucrose, raffinose, and stachyose), and storage carbohydrate (fructans in RCG) composition for the biomass are summarized in Table 1 for RCG. Ash content and lignin content of these samples were reported elsewhere *(11)*.

AFEX Pretreatment

AFEX, a physio-chemical pretreatment, is a process in which concentrated ammonia is used to treat biomass at a desired residence time for a given temperature, moisture content, and ammonia loading. The pressure is released rapidly causing the biomass to expand. The pretreated biomass is kept in a fume hood overnight to remove ammonia. After the ammonia has evaporated, the biomass is ready for hydrolysis. More details about the AFEX reactors are available elsewhere *(10,12).* For this work, AFEX pretreatment conditions varied were temperature (80–120°C) and ammonia loading (0.8, 1.0, and 1.2 kg/1 kg dry biomass) at fixed 60% moisture content (dry weight basis). The moisture condition was fixed at 60% based on our experience in pretreating other grass materials like corn stover and rice straw using AFEX *(13,14).* Usually, it takes 30–40 min to pretreat 25.0 g of biomass from start to the end. It has to be noted that AFEX is a dry-to-dry process and the glucan content remains the same even after pretreatment unless the sample is washed before hydrolysis. Separate glucan analyses were done using National Renewable Energy Laboratory (NREL, Golden, CO) protocol *(11)* when the samples were washed.

Enzymatic Hydrolysis

AFEX-treated and untreated materials were washed with water (1.0 g/10 mL water) according to the NREL protocol. Enzymatic hydrolysis was then done using NREL standard procedure (LAP 009) at 15 FPU cellulase/g glucan. Each experiment was done in duplicate *(13,14)*. Hydrolysis was performed simultaneously for each AFEX-treated and untreated sample using Spezyme CP cellulase enzyme (59 FPU/mL and 142 mg/mL; Genencor International, Inc., Rochester, NY) and Novozyme 188 β-glucosidase (64 para-nitro-phenyl glucoside unit [pNPGU]; cellobiase; Sigma, St. Louis, MO). The Spezyme CP cellulase mixture also contains a small amount (about 1% by mass) of hemicellulase activity. The hydrolysis was done using 1% glucan loadings (0.15 g glucan and 15 mL total sample volume) in capped vials, which were placed in an incubator at 50°C, 90 rpm

Table 1
Carbohydrate Composition of Biomass Forage Samples

	Cell wall structural carbohydrates								Soluble carbohydrates						Total carbohydrates	
Biomass	Glc	Xyl	Ara	Gal	Man	Rha	Fuc	UA	Glc	Fru	Suc	Raf	Sta	Storage[a]	NSC	SC
RCG[b]																
VRCG	209	117	30	16	6	1	1	22	4	5	69	3	0	35	116	402
SRCG	265	163	28	13	6	1	1	21	2	12	30	0	1	54	99	498

NSC, nonstructural carbohydrates; SC, structural carbohydrates; Glc, glucose; Xyl, xylose; Ara, arabinose; Gal, galactose; Man, mannose; Rha, rhamnose; Fuc, fucose; UA, uronic acids; Fru, fructose; Suc, sucrose; Raf, raffinose; Sta, stachyose; RCG, reed canarygrass; VRCG, vegetative stage; SRCG, seed stage.

From ref. *10*.

[a]Storage carbohydrate for SWG was starch. RCG had fructans in the vegetative sample (37.0 g/kg).

[b]Data for RCG are for whole herbage (g/kg DM).

for 168 h as described in the procedure. Samples of 1 mL were taken periodically at 24, 72, and 168 h interval, respectively. Each collected sample was centrifuged and filtered through a 0.2 µm syringe filter containing a nylon membrane into a high-performance liquid chromatography (HPLC) vial and frozen at –20°C, until further analysis.

In some experiments, Multifect 720 xylanase (42.0 mg/mL; Genencor International, Rochester, NY) was added to the cellulase and β-glucosidase mixtures to determine the effect of xylanase on glucose and xylose yields. Each xylanase was loaded at 10–50% (by weight) of the cellulase loading. The samples were hydrolyzed for 168 h and analyzed for glucose and xylose conversions using HPLC. Control experiments were done without adding enzymes to determine the amount of free sugars in both growth stages of RCG. These free sugars were subtracted from the results for enzymatically hydrolyzed samples. Thus, the enhanced sugar yields reported here result from hydrolysis of structural carbohydrates.

Analytical Methods

The HPLC system consisted of Waters (Milford, MA) Pump and Waters 410 refractive index detector, and an Aminex HPX-87P carbohydrate analysis column (Bio-Rad, Hercules, CA) equipped with a deashing guard cartridge (Bio-Rad). Degassed HPLC grade water was used as the mobile phase at 0.6 mL/min at a column temperature of 85°C. The injection volume was 20 µL with a run time of 20 min. Mixed sugar standards were used for quantification of cellobiose and other monosaccharides (glucose, xylose, galactose, arabinose, and mannose) in the samples. More details about the glucose and xylose conversions are given in NREL protocol (LAP009).

Results and Discussion

Vegetative RCG

Figure 1A shows glucose and xylose yields for VRCG (untreated and treated) after 168 h of hydrolysis. Percent conversions ranged from 48 to 86% for glucan and 33 to 78% for xylan based on cell wall composition. Temperature affects the amount of ammonia vaporized during the explosive flash (for AFEX) *(7)*. It appears that at the lowest temperature (80°C) tested, more ammonia must be used (>1.2 kg ammonia : 1 kg dry matter [DM]) to achieve higher glucose conversion, whereas at a higher temperature (100°C), less ammonia (1 kg ammonia : 1 kg DM) achieved higher glucose conversion. More ammonia vapors flash at higher reactor temperatures, causing greater disruption of the fibrous structure *(7)*. Temperature also likely influences the nature of ammonia's reaction with lignin and the extent of alkaline hydrolysis of hemicellulose. Elevating the temperature beyond 100°C reduced conversion yields for both glucose and xylose. Experimental work to better understand possible deleterious effects of pretreatment done at high temperature is underway. The most effective AFEX condition for

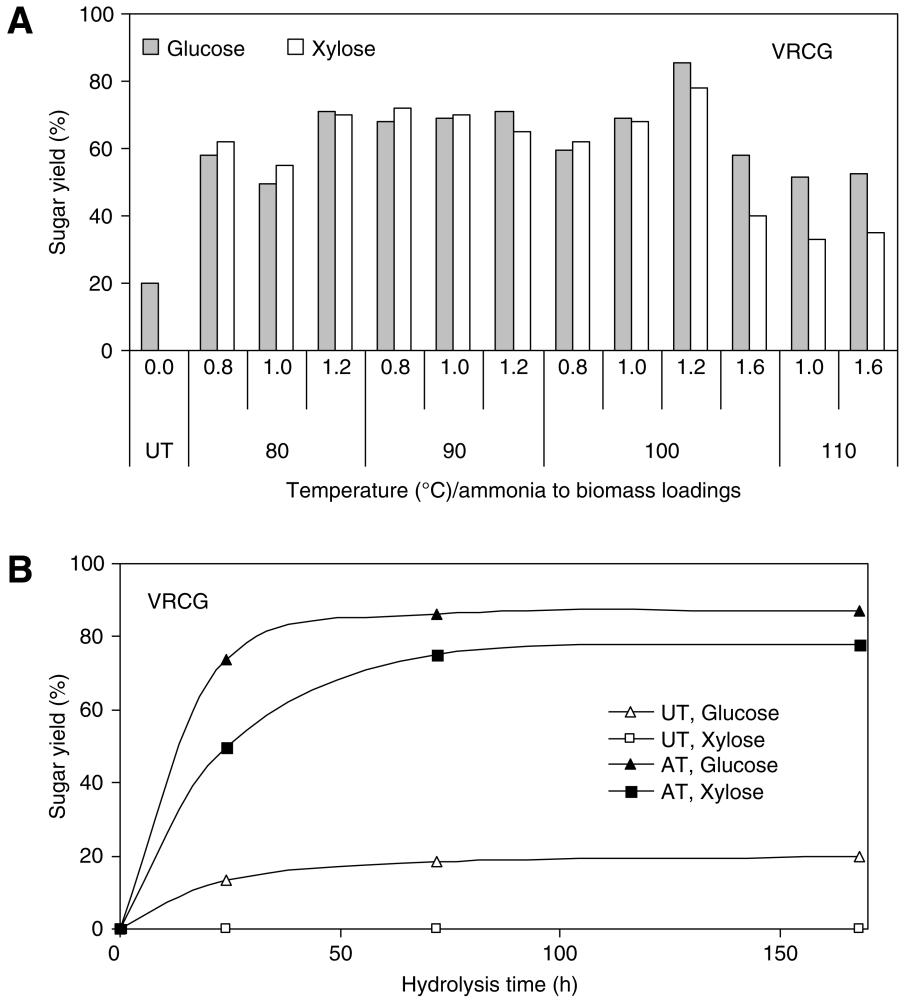

Fig. 1. Glucan and xylan conversions for untreated and AFEX-treated VRCG. **(A)** AFEX pretreatment screening was done with 60% moisture in the biomass and by varying the temperature and ammonia-to-biomass loadings. Hydrolysis experiments were done using 15 FPU cellulase/g of glucan and sugar concentration measured at 168 h using HPLC. The sugar conversions are an average of two independent analyses and are consistent with an error range of ±2%. **(B)** Time-course experiments were done to measure glucose and xylose yield for the best AFEX-treated conditions (100°C, 1.2 : 1 ammonia-to-biomass, 60% moisture).

VRCG was found to be 100°C, 60% moisture, 1.2 kg : 1 kg (ammonia : DM), which yielded 86% of theoretical glucose based on cell wall glucan and 78% xylose conversion based on measured cell wall xylan (Table 1).

Although temperature and ammonia loading were increased beyond our usual AFEX pretreatment conditions for SWG *(12)* to explore the possibility of achieving higher yields for both glucose and xylose, VRCG did not behave well under such conditions. Nonetheless, AFEX-treated VRCG

increased glucan conversion by 66 percentage points (pp) and 78 pp for xylan over conversions observed for untreated VRCG.

Figure 1B shows the hydrolysis profile for AFEX-treated and untreated VRCG at an enzyme level of 15 FPU cellulase/g glucan. Results from both the most effective and least effective AFEX treatment conditions for VRCG and untreated VRCG are plotted herein. It is interesting to note that the xylose content of untreated VRCG is completely resistant to enzymatic hydrolysis, whereas about 20% glucose conversion occurs at 168 h hydrolysis. With AFEX pretreatment, more structural carbohydrates are apparently exposed allowing the enzymes to better digest VRCG, increasing both glucose and xylose conversions. For example, after only 24 h of hydrolysis, the most effective AFEX condition observed for VRCG (100°C, 60%, 1.2 kg ammonia : 1 kg DM) generated approx 60 pp more glucose and 50 pp more xylose than did untreated VRCG. By 72 h of hydrolysis, the treated VRCG produced 68 pp more glucose and 75 pp more xylose than did untreated material. The data show that by the end of hydrolysis (for the most effective AFEX condition), glucose and xylose yields increased by 68 and 78 pp, respectively, with AFEX pretreatment. The hydrolysis profile also summarizes the time needed to completely digest the samples. Figure 1B shows that at the most, effective AFEX condition for VRCG reached a peak for glucose and xylose conversion after 72 h hydrolysis.

Seed Stage RCG

Hydrolysis of SRCG (including untreated SRCG) was observed by measuring glucose and xylose conversions under various pretreatment parameters. Figure 2A displays the 168 h conversions for SRCG. The pretreatment effectiveness improved at each temperature with increasing ammonia loading up to 100°C. However, after 100°C, the conversion levels begin to decrease with increasing ammonia loading. Ammonia can react with lignocellulosics by ammonolysis of the ester crosslinks of some uronic acids with the xylan units *(15),* and by cleaving the bond linkages between hemicellulose and lignin *(16).* It is possible that extra liquid ammonia plasticizes the cellulose and thereby reduces the disruptive effect of sudden pressure release *(17).* In Fig. 2A, SRCG treated at different conditions (90°C, 60%, and 1.2), (100°C, 60%, and 0.8), and (100°C, 60%, and 1) provides 89% conversion for glucose and 81% for xylose, respectively.

Figure 2B illustrates the hydrolysis profile for the most and least effective AFEX conditions for SRCG and untreated SRCG at 15 FPU cellulase/g glucan enzyme loadings. It is apparent from Fig. 2B that the structure of SRCG is disrupted by AFEX allowing nearly 4.4 times more glucose and a 50 pp increase in xylose yield with the most effective AFEX condition compared with untreated SRCG at the end of 24 h. At 72 h of hydrolysis, glucose and xylose conversions were consistent with 24 h results for the most effective AFEX condition for SRCG. However, the glucan conversion for the untreated sample increased by 9 pp. By the end of 168 h of

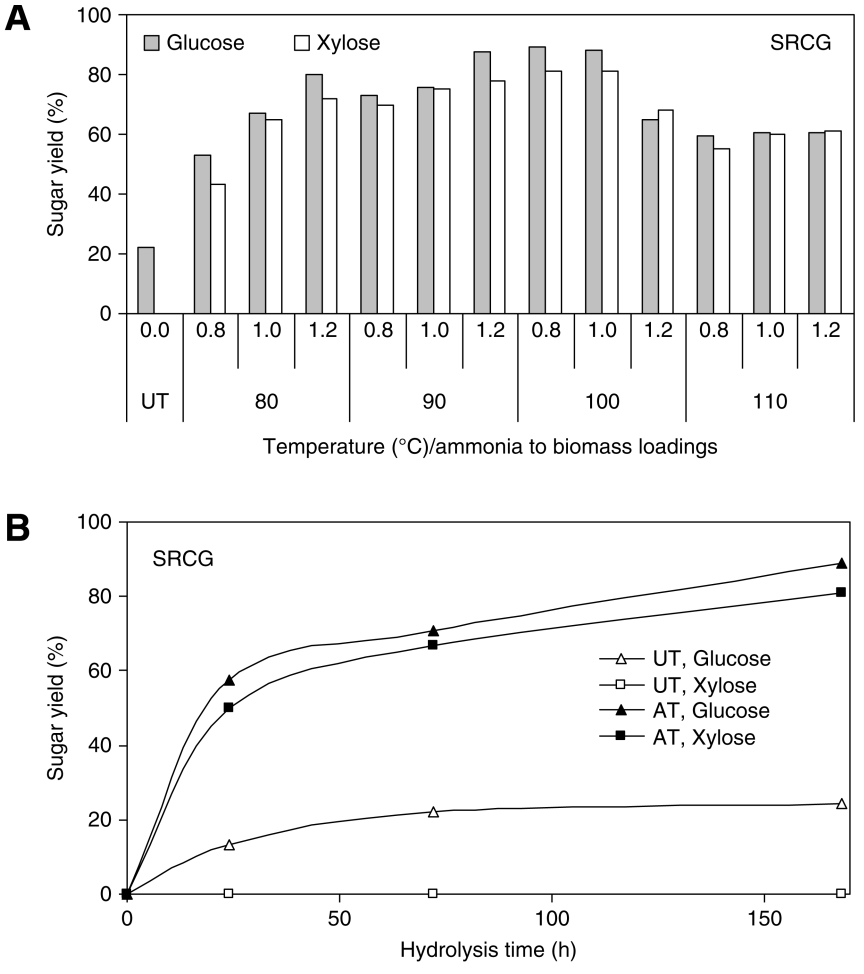

Fig. 2. Glucan and xylan conversions for untreated and AFEX-treated SRCG. **(A)** AFEX pretreatment screening was done with 60% moisture in the biomass and by varying the temperature and ammonia-to-biomass loadings. Hydrolysis experiments were done using 15 FPU cellulase/g of glucan and sugar concentration measured at ses. **(B)** Time-course experiments were done to measure glucose and xylose yield for the best AFEX-treated conditions (100°C, 1 : 1 ammonia-to-biomass, 60% moisture).

hydrolysis, conversion of AFEX-treated SRCG increased 18 pp and 14 pp for both glucan and xylan, respectively, compared with the 72 h results. Just 2 pp improvement in glucan conversion was noticed in the untreated sample. Glucose and xylose yields show 65 pp and 81 pp, respectively, enhancement for AFEX-treated SRCG (100°C, 60%, and 0.8) over the untreated sample. Again, as observed for VRCG, the xylan portion of the untreated SRCG is completely resistant to enzymatic hydrolysis. The maximum xylose conversion for the most effective AFEX condition is obtained at 168 h, producing only 80% of theoretical conversion.

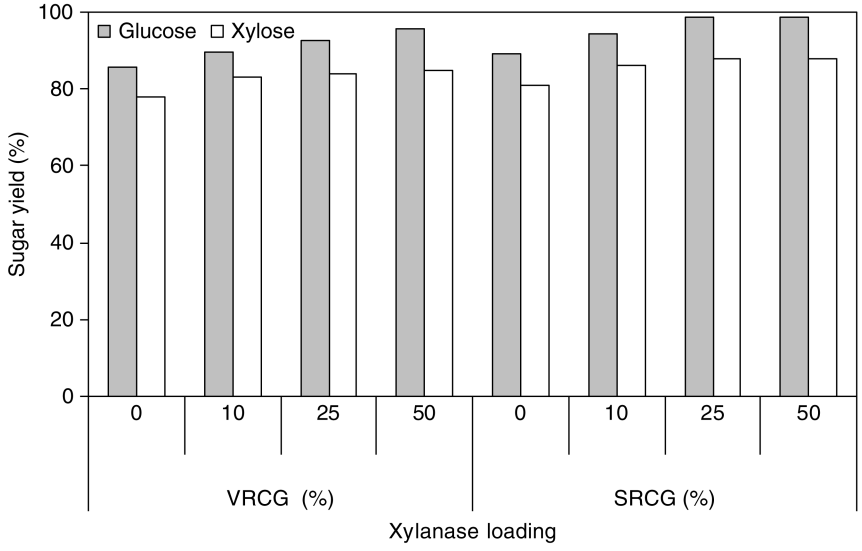

Fig. 3. Effect of xylanase supplementation along with cellulase (15FPU/g of glucan), Glucan, and xylan conversions for untreated and AFEX-treated VRCG and SRCG. Hydrolysis experiments were done using 15 FPU cellulase/g of glucan with xylanase (protein concentration 42.0 mg/mL) supplementation (10, 25, or 50%) of the total milligrams of cellulase protein (protein concentration 142 mg/mL). The sugar concentration was measured at 168 h using HPLC.

Cellulase and Xylanase Combination

Samples treated at the most effective AFEX condition found for each of the materials were enzymatically hydrolyzed for 168 h with a combination of Spezyme CP cellulase and Multifect 720 xylanase at 15 FPU cellulase/g glucan. The AFEX-treated samples were tested at various xylanase loadings (10, 25, and 50%, by weight, respectively) of the cellulase loading. Figure 3 shows the increase in glucose and xylose conversions attained for the most effective AFEX conditions for VRCG and SRCG for this specific enzyme combination. Multifect 720 xylanase was shown to enhance both glucose and xylose conversions for all the three samples.

Unlike the glucose conversion shown in Fig. 3, the xylose conversions steadily increased with increasing xylanase loading. At a 10% xylanase loading, both maturity stages for RCG produced about 4 pp higher glucan and xylan conversions. At 25% xylanase loading, the glucan conversion for RCG nearly doubled, whereas the xylan conversion gradually increased by 1 and 2 pp for VRCG and SRCG, respectively. As observed at 50% xylanase loading, VRCG continued to progress, whereas SRCG showed no change with the increase of xylanase compared with 25% xylanase loadings. We conclude that the commercial xylanse is not particularly suited to hydrolyze the hemicellulose in RCG, but that even small increases in xylan conversion promote significant increases in glucan conversion.

Discussion

RCG show enhanced glucan and xylan conversions following AFEX pretreatment. The maturity of the materials apparently influenced the conversion of sugars. In a previous study, it was shown that the maturity level for RCG and SWG affected the lignin concentrations, which could explain the rapid digestion observed for vegetative stage compared with seed stage *(11)*. Our results further confirm their hypothesis. Dien et al. *(11)* provided the lignin content as 109 (g/kg DM) for VRCG and 148 (g/kg DM) for SRCG. Consequently, the lignin content is lower for VRCG (which is an earlier stage of RCG) compared with those of SRCG, and might be a major reason why higher percent conversions were achieved with VRCG than with SRCG. Lignin concentrations also increased for the more mature samples *(11,18,19)*. Therefore, more mature stages of RCG gave higher lignin content as well as higher concentrations in structural and nonstructural carbohydrates *(11)*. Because the lignin content is higher for more mature materials, hydrolyzing the sugars from these materials would probably require more enzyme or more extreme pretreatment conditions to achieve higher conversions. A material in its earlier stage growth with lower lignin content would be expected to show higher conversions with less hydrolysis time (or digestion), as observed here *(11,20)*.

The protein content in the vegetative stage is greater compared with the seed stage. Protein could bind degradation products produced during AFEX, and hence, reduce possible enzyme inhibition. Our results also show that xylanase formulations need to be improved in order to get maximal conversion of the hemicellulose to xylose for RCG and likely for other grass materials. However, even small increases in xylan conversion appear to greatly increase glucan conversion. Both the glucan and xylan conversions reported in the paper are understated owing to uncertainties associated with glucan estimation after washing. That is, the actual conversions are somewhat higher than we claim here. More experiments are under way to understand the degradation products formed during pretreatment. Removal of these degradation products may enhance enzyme activity and reduce possible inhibition of microbes in downstream processing.

Acknowledgments

We sincerely thank our colleagues from the USDA: Drs. Bruce Dien, Hans Jung, Kenneth Vogel, Michael Casler, JoAnn Lamb, Loren Iten, Robert Mitchell, and Gautum Sarath for supplying two different stages of RCG and for the detailed analytical data on these materials that allowed us to perform this work.

References

1. Dale, B. (2002), *Encyclopedia of Physical Science and Technology,* 3rd ed. vol. 2, pp. 141–157.
2. Gray, K. A., Zhao, L., and Emptage, M. (2006), *Curr. Opin. Chem. Biol.* **10,** 141–146.
3. Dale, B. (1987), *Trends Biotechnol.* **5,** 287–291.
4. Dale, B., Leong, C., Pham, T., Esquivel, V., Rios, I., and Latimer, V. (1996), *Bioresour. Technol.* **56,** 111–116.
5. Williams, K. (1995), http://www.agron.iastate.edu/moore/434/chapter7.htm.
6. Mes-Hartee, M., Dale, B. E., and Craig, W. (1998), *Appl. Microbiol. Biotechnol.* **29,** 462–468.
7. Mosier, N., Wyman, C., Dale, B., et al. (2005), *Bioresour. Technol.* **96,** 673–686.
8. Lemus, R. E., Brummer, C., Moore, K. J., Molstad, N. E., Burras, C. E., and Barker , M. F. (2002), *Biomass Bioenergy* **23,** 433–442.
9. Dale, B., Henk, L., and Shiang, M. (1985), *Dev. Ind. Microbiol.* **26,** 223–233.
10. Chundawat, S. P. S., Venkatesh, B., and Dale, B. E. (2007), *Biotechnol. Bioeng.* **96,** 219–231.
11. Dien, B., Jung, H., Vogel, K., et al. (2006), *Biomass Bioenergy* **30,** 880–891.
12. Alizadeh, H., Teymouri, F., Gilbert, T. I., and Dale, B. E. (2005), *Appl. Biochem. Biotechnol.* **121–124,** 1133–1141.
13. Gollapalli, L. E., Dale, B. E., and Rivers, D. M. (2002), *Appl. Biochem. Biotechnol.* **98–100,** 23–35.
14. Teymouri, F., Laureano-Perez, L., Alizadeh, H., and Dale, B. E. (2004), *Appl. Biochem. Biotechnol.* **113–116,** 951–963.
15. O'Connor, J. J. (1972), *Tappi* **55,** 353.
16. Wang, P., Bolker, H., and Purves, C. (1967), *Tappi* **50,** 123–124.
17. Rowland, S. (1975), *Biotechnol. Bioeng.* **21,** 1031–1042.
18. Holtzapple, M. T., Jun, J. H., Ashok, G., Patibandla, S., and Dale, B. E. (1991), *Appl. Biochem. Biotechnol.* **28–29,** 59–72.
19. McLaughlin, S. B., Bransby, D. I., and Parrish, D. (1994), (http://www.osti.gov/energy citations/servlets/purl/10189529-mvgLAy/webviewable/10189529.pdf) (accessed date Feb. 2007).
20. Fan, L., Lee, Y. Y., and Beardmore, D. (1980), *Biotechnol. Bioeng.* **22,** 177–199.