0273-2289/00/84–86/0163/$14.25

Optimizing Ammonia Pressurization/Depressurization Processing Conditions to Enhance Enzymatic Susceptibility of Dwarf Elephant Grass

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Abstract

An ammonia pressurization/depressurization process was investigated to evaluate the potential of producing reducing sugars from dwarf elephant grass, a warm-season forage. Moisture, temperature, and ammonia loading affected sugar yield ($p < 0.0001$). At optimal conditions, ammonia processing solubilized 50.9% of the hemicellulose and raised the sugar yield (percentage of theoretical) from 18 to 83%. Glucose and xylose production were increased 3.2- and 8.2-fold, respectively. The mild processing conditions of the ammonia treatment (90–100°C, 5 min), the low enzyme loading (2 international filter paper units/g), and the short hydrolysis time (24 h), greatly enhance the potential of using forages to produce sugars valuable for several applications.

Index Entries: Ammonia; dwarf elephant grass; enzymatic hydrolysis; sugars.

Introduction

The bioconversion of agricultural residues (cereal straws, stovers, and so on) has received considerable attention. However, relatively few studies

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have been conducted on forages. The main purpose of the studies has been to investigate the production of sugars for fuels, mainly ethanol, by fermentation processes *(1,2)*, and for animal feeding *(3,4)*. Both acid and enzymatic hydrolysis have been used, but the latter might be preferred owing to milder conditions and fewer degradation products. Currently several stable cellulolytic enzymes with high activity are on the market. However, fibers are resistant to enzymatic hydrolysis owing to physical and chemical constraints of the plant cell wall, and sugar yields would therefore be rather small. When forages such as Bermuda grass, smooth bromegrass, switchgrass, and alfalfa were subjected to cellulase hydrolysis *(5,6)*, saccharification was small compared to that occurring in the rumen of animals, unless the forages were subjected to additional digestion with acid pepsin or neutral detergents *(7,8)*. These digestions are suitable as in vitro digestibility tests, but they are not appropriate for industrial processing. However, sugar production by enzymatic hydrolysis of forages can be greatly enhanced if pretreatments are applied to forages *(9)*; alkali and steam explosion treatments are the most commonly used.

Ammonia treatments (30 min) have been found to be effective for increasing the susceptibility of coastal Bermuda grass, switchgrass *(1,10)*, and corn fiber *(2)* to enzymatic hydrolysis. Theoretical conversions of fibers into sugars up to 90% have been attained using low enzyme doses (5–10 IU/g) and a 24-h incubation. In addition, ammonia reactor treatments have not produced materials toxic for fermentation processes *(11)*.

In the present study, ammonia at ammonia pressurization/depressurization (PDA) treatment conditions was used to improve the susceptibility of fibers to enzyme degradation *(12)*. First of all, PDA treatments solubilize a fraction of the hemicellulose (about 50%) and lignin (up to 30%) *(13)*. Because of the action of ammonia, enhanced by moisture and the temperature rise produced by its exothermic solubilization in water, and the sudden depressurization in PDA treatments, susceptibility of fibers to enzymatic hydrolysis greatly increases. Final temperatures are well above freezing (about 40°C), contrary to ammonia freeze explosion (AFEX), in which freezing is part of the process. Materials only need to be shredded, not grinded as in AFEX. Ammonia has reacted in the liquid phase, and therefore moisture is required in the process. Efficiency is such that the expected results occur in 2–5 min. Cellulose saccharification produces glucose. In addition to glucose, forages may contain other sugars such as fructose, galactose, and sucrose, which are also excellent substrates for fermentation. On the other hand, xylose and arabinose, the main products of hemicellulose hydrolysis, can also be metabolized even to ethanol *(14)*.

The species used in the present study was dwarf elephant grass (DEG), a tropical perennial bunchgrass released in 1988 by the University of Florida *(15)*. The leaf content comprises 74% of plant dry matter (DM) compared with only 46% for tall elephant grass *(16)*. DEG is relatively cold tolerant, and although a northern limit has not been established, it is expected to be well adapted in extreme southern Georgia, in most of Florida, and in areas of the

subtropics and tropics where tall elephant grass grows well *(15)*. DEG is widely distributed in Venezuela and, together with guinea grass, comprises more than 80% of the grasses grown in the western part of that country *(17)*.

The main objective of this study was to assess the effect of PDA treatment conditions on sugar production by enzymatic hydrolysis of DEG. In addition, sugar profiles were determined to assess the selectivity of the ammonia treatment for forage fiber components. Effects on carbohydrate fraction were also determined to test the efficacy of the treatments prior to hydrolysis.

Materials and Methods

PDA Treatment

A laboratory-scale ammonia reactor unit consisting of a 4-L reactor with appropriate support equipment was used for the treatment of DEG. DEG (*Pennisetum purpureum* Sum.cv.Mott) obtained from the Texas Experiment Station (College Station, TX) was used in these studies. It was ground to 20 mesh and kept under refrigeration until use. Liquid anhydrous ammonia was added to 80-g samples, and temperature was rapidly raised to the desired temperature. After treatment time, pressure was suddenly released and samples were allowed to air-dry overnight. Ammonia loadings (*r*) of 0.5, 1.0, and 1.5 g of ammonia/g of DM, delivered in 4 min, were tested. Experiments were carried out at two temperatures (*T*), 75 and 90°C, and two moisture contents (*M*), 30 and 60% (wet weight basis [w.b.]). PDA treatments are labeled as *r-M-T* treatments throughout the article. Neutral detergent fiber (NDF), acid detergent fiber, and acid detergent lignin were determined in triplicate to estimate cellulose, hemicellulose, and lignin *(18)*.

Enzymatic Hydrolysis

Untreated and the best PDA-treated samples (lowest hemicellulose content) were subjected to enzymatic hydrolysis by cellulase (Spezyme CP; Genencor, Rochester, NY) and cellobiase (Novozym 188; Novo Nordisk, Franklinton, NC). The enzymatic hydrolyses were performed with cellulase loadings of 1, 2, and 5 IU/g of biomass during 72 h of digestion. Cellobiase loading was kept at a 5.68 cellobiase units (CBU)/IU (cellobiase/cellulase) ratio. Hydrolysis was carried out at a solids loading of 5% (w/v) in 100 mL of 0.05 *M* citrate buffer (pH 4.8) with sodium azide added (0.15%) for preservation. Duplicate 500-mL Erlenmeyer flasks containing the samples were placed in an INNOVA 4300 incubator shaker (New Brunswick Scientific, Edison, New Jersey) at 50°C at 100 rpm. Samples were filtered through cheesecloth, cooled, centrifuged at 3000 rpm (760*g*), and filtered on 0.22-μm Millipore nylon membranes prior to sugar analysis. Reducing sugar production was determined as glucose equivalent on the filtrates at 0, 3, 6, 12, 24, 48, and 72 h of digestion using the dinitro salicylic acid (DNS) method *(19)*. From this experiment, based on reducing sugar production, optimal enzyme loading and hydrolysis time were chosen to test the rest of the samples.

Untreated and all treated samples were subjected to enzymatic hydrolysis by cellulase (Spezyme CP), cellobiase (Novozym 188), and hemicellulase (Multifect XL; Genencor, Rochester, NY), at optimal cellulase loading and hydrolysis time. Hemicellulase was included to increase the production of xylose. Cellulase, cellobiase, and hemicellulase loadings were kept at 2 IU/g of DM, 11.4 CBU/g *(10)*, and 2 IU/g, respectively. Hydrolysis conditions were the same as those indicated previously. Sugar production was determined by measuring reducing sugars with the DNS method *(19)* at zero time and at 24 h. Zero-time determinations for all runs were made after 5 h of incubation with no enzymes added. Sugars present initially in the enzyme solutions were subtracted from the original sugar yields to determine actual sugar production (3–6 mg/g of DM). Sugars originally present in the substrates at t = 0 h were subtracted from 24-h yields to estimate net sugar yields. Sugar yield was also expressed as percentage of theoretical conversion taking into account both cellulose and hemicellulose content of untreated DEG. Degrees of cellulose and hemicellulose conversion were estimated based on the concentrations of glucose, and pentoses plus galactose in the hydrolysate, respectively. Sugar profiles for 0- and 24-h hydrolysis were determined by high-performance liquid chromatography (HPLC) analysis, using a Waters 600 delivery system (Waters, Milford, MA) equipped with a Bio-Rad (Bio-Rad, Cambridge, MA) Aminex HPX-87P column (300 × 7.8 mm) and a Waters 410 refractive index detector. Samples from the filtrates were injected (20 μL) and eluted with water (0.6 mL/min) at 85°C. Standard sugars were from Sigma (St. Louis, MO).

The results of fiber analysis (solubles, hemicellulose, and cellulose) and reducing sugar yield were analyzed using general linear models (GLM) procedures of statistical analysis system (SAS) *(20)*. An analysis of variance was applied to each of the studies, and significance of the PDA treatment compared to untreated, differences among PDA treatment conditions, effects of main variables (ammonia loading, moisture content, and temperature), and two- and three-way interactions were investigated. Standard errors of the means (SEM) were calculated and included in the figures.

Cellulase activities were confirmed by the filter paper method *(21)*. Spezyme CP had 115.6 international filter paper units (IFPU)/mL. These data were used with information on cellobiase and xylanase activity from the manufacturers to set the enzyme dosages.

Results and Discussion

Effect on Carbohydrate Fraction

Earlier work showed that treatments causing the greatest increase in NDF solubles content and the greatest decrease in hemicellulose concentration also caused the highest digestibility measured as ruminal *in situ* digestibility *(13)*. Therefore, it is expected that is also related to an increase in fiber susceptibility to enzymatic hydrolysis. Untreated DEG had 31.4% solubles (NDF solubles), 33.0% hemicellulose, 32.0% cellulose, and 3.7% lignin

Table 1
Effects of PDA Treatment on Carbohydrate Fraction

Variable	Control value (% DM)[a]	Optimal treatment condition (*r*-*M*-*T*)[b]	Treatment value (% DM)	Net change (%)
Solubles	31.4	1-60-90	48.16	53.3
Hemicellulose	33.0	1-60-90	16.20	50.9
Cellulose	32.0	1-60-90	32.89	2.8

[a]Values of untreated DEG were significantly different from treated ($p < 0.0001$), except for cellulose.
[b]*r*, ammonia loading (g NH_3/g DM); *M*, moisture (% w.b.); *T*, temperature (°C).

Table 2
Statistical Significance of Main Effects and Interactions

Source	Probability value		
	Solubles	Hemicellulose	Cellulose
Untreated vs treated	0.0001	0.0001	0.0002
Among PDA treatments	0.0001	0.0001	0.0001
Main effects			
r	0.0001	0.0001	0.2029
M	0.0001	0.0001	0.0001
T	0.0001	0.0001	0.1821
Interactions[a]			
$r \times M$	0.0047	0.9981	0.0159
$r \times T$	0.0001	0.0026	0.0285
$M \times T$	0.0003	0.5571	0.0291
$r \times M \times T$	0.5230	0.1745	0.0079

[a]*r*, Ammonia loading (g NH_3/g DM); *M*, moisture (% w.b.); *T*, temperature (°C).

(Table 1). There were significant differences between untreated and PDA-treated DEG for solubles ($p < 0.0001$), hemicellulose ($p < 0.0001$), and cellulose ($p < 0.0002$). In addition, there were significant differences ($p < 0.0001$) among PDA treatments (Table 2).

Figure 1A–C presents the main effects of *r*, *M*, and *T* on carbohydrate fraction of DEG. Solubles (Fig. 1A) of the untreated DEG increased with ammonia loading up to 1.0 ($p < 0.0001$). Solubles are solubilized fractions of hemicellulose (hemicellulodextrins), which are more rapidly hydrolyzed than the original insoluble hemicellulose *(22)*. Solubles at $r = 1.5$ decreased, suggesting that excessive ammonia loadings may cause Maillard-type condensation reactions that reduce the concentration of reducing sugars *(23)*. The best treatment, 1-60-90, increased solubles from 31.37 to 48.16%, an increase in solubles of 53.3% (Table 1). Hemicellulose concentration decreased from 32.95% in untreated DEG to 16.20% in the best treatment (1-60-90), which represents a 50.9% reduction. This suggests that solubles

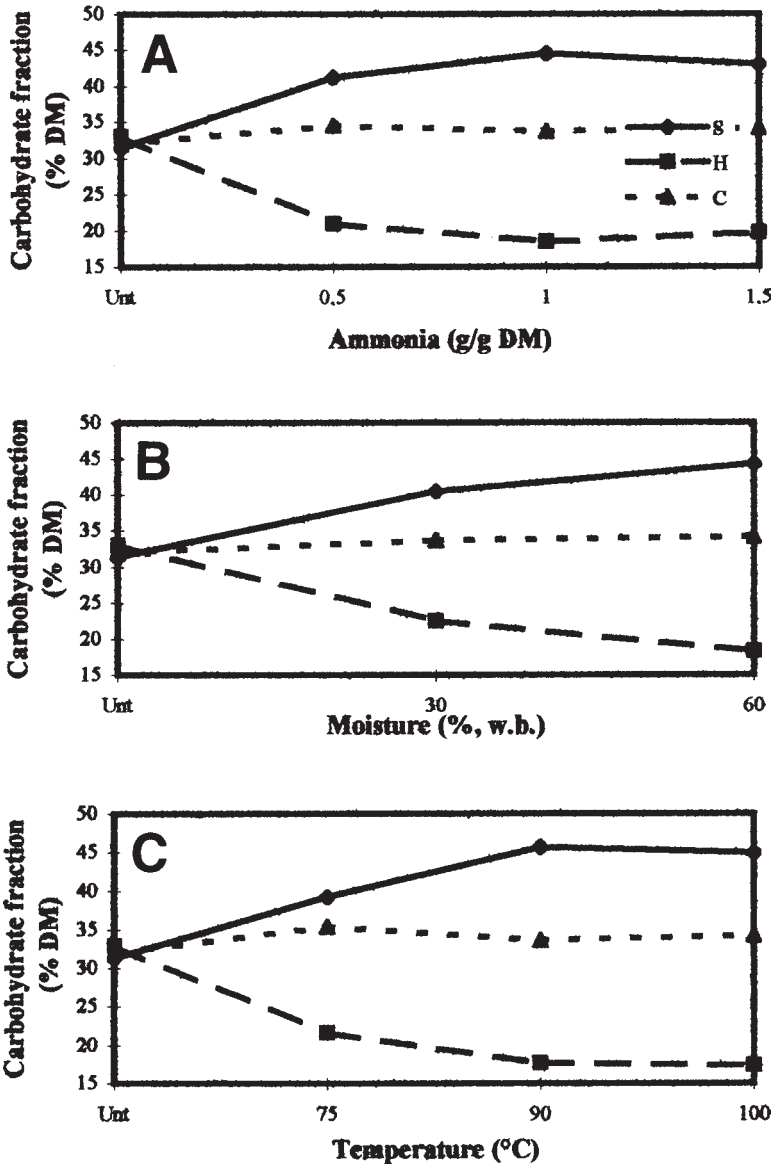

Fig. 1. Carbohydrate fractions of untreated (Unt) and PDA-treated DEG, at selected **(A)** ammonia loadings, **(B)** moisture contents, and **(C)** temperatures. S, solubles; H, hemicellulose; C, cellulose. SEM values: (A) S = 0.30, H = 0.29, C = 0.22; (B) S = 0.30, H = 0.29, C = 0.22; (C) S = 0.31, H = 0.30, C = 0.23.

are likely produced by partial hydrolysis *(24)* and solubilization of hemicellulose by the alkali treatment *(25)*.

Hemicellulose solubilization, together with a partial lignin solubilization, greatly enhances cellulose saccharification. In fact, the opening of the cellulose matrix is facilitated by the absence of these physical-chemical barriers. On the other hand, a significant increase ($p < 0.05$) of hemicellulose

for r = 1.5 suggests that condensation reactions form compounds that appear as hemicellulose measured by the Goering and Van Soest's method *(18)*. Cellulose remained approximately unchanged. An apparent increase in cellulose concentration with the PDA treatment may be owing to the method used to estimate it *(18)*.

Figure 1B shows the significant effect of moisture on solubles and hemicellulose of DEG. The higher the moisture, the higher the solubles and the lower the hemicellulose. Moisture appears to facilitate ammonia penetration of the biomass for reaction, and since ammonia dissolution in water is exothermic, the rate of reaction increases. Moisture contents below 30% were not tested because they were not successful in increasing solubles of other materials such as rice straw, in which 60% moisture had a greater effect than 30% *(12)*.

Figure 1C shows a significant increase ($p < 0.0001$) in solubles and a decrease in hemicellulose when DEG is PDA-treated at 75 and 90°C. When temperature was raised to 100°C, no further improvement was attained.

Figure 2 shows significant two-way interactions for r, M, and T affecting solubles content of DEG. Three-way interactions were nonsignificant ($p > 0.05$). Figure 2A shows that at 30% moisture, solubles increased with increasing r, but at 60%, an r of 1.5 had the opposite effect since solubles decreased compared to the treatment of 1.0 r ($p < 0.05$). Increasing r from 0.5 to 1.0 had a much greater effect at 60% M than at 30%. Figure 2B shows the enhancing effect in solubles of increasing moisture content at 90°C, but not at 75°C, since treatments of 30% M-75°C and 60% M-75°C were not significantly different ($p > 0.05$). In addition, increasing temperature from 90 to 100°C at 60% M did not increase solubles any further. High r-T combinations (Fig. 2C) were deleterious since solubles produced by the 1.0 r-100°C treatment were not higher than those for the 1.0 r-90°C treatment ($p > 0.05$), and those produced by 1.5 r-100°C decreased compared to 1.5 r-90°C ($p < 0.05$). It appears that high r-M-T combinations tend to produce overreaction with an apparent reduction in solubles and apparent increase in hemicellulose. Only $T \times r$ and $M \times r$ interactions were significant for hemicellulose.

Figure 3A shows the deleterious effect of high $M \times r$ combinations as an increase in hemicellulose for the 1.5 r-60% treatment compared to the 1.0 r-60% treatment, which was the best combination. At 30% M, 1.5 and 1.0 r treatments were not significantly different ($p > 0.05$). Figure 3B indicates an increase in hemicellulose for 1.5 r compared to 1.0 r, at all temperatures. Interestingly, hemicellulose was higher for 75 and 100°C than for 90°C. At 75°C, hemicellulose was high owing to a poor effect of ammonia at low temperatures, whereas at 90°C, hemicellulose decreased but increased again at 100°C, probably because of condensation reactions. Figure 3B, however, clearly shows that an r of 1.5 should not be used in DEG in this range of temperatures and moistures, because it has no effect (comparing treatments 1.5 r-90°C and 1.0 r-90°C), or has a deleterious effect, as previously noted. Three- and two-way interactions were not significant for cellulose ($p > 0.05$).

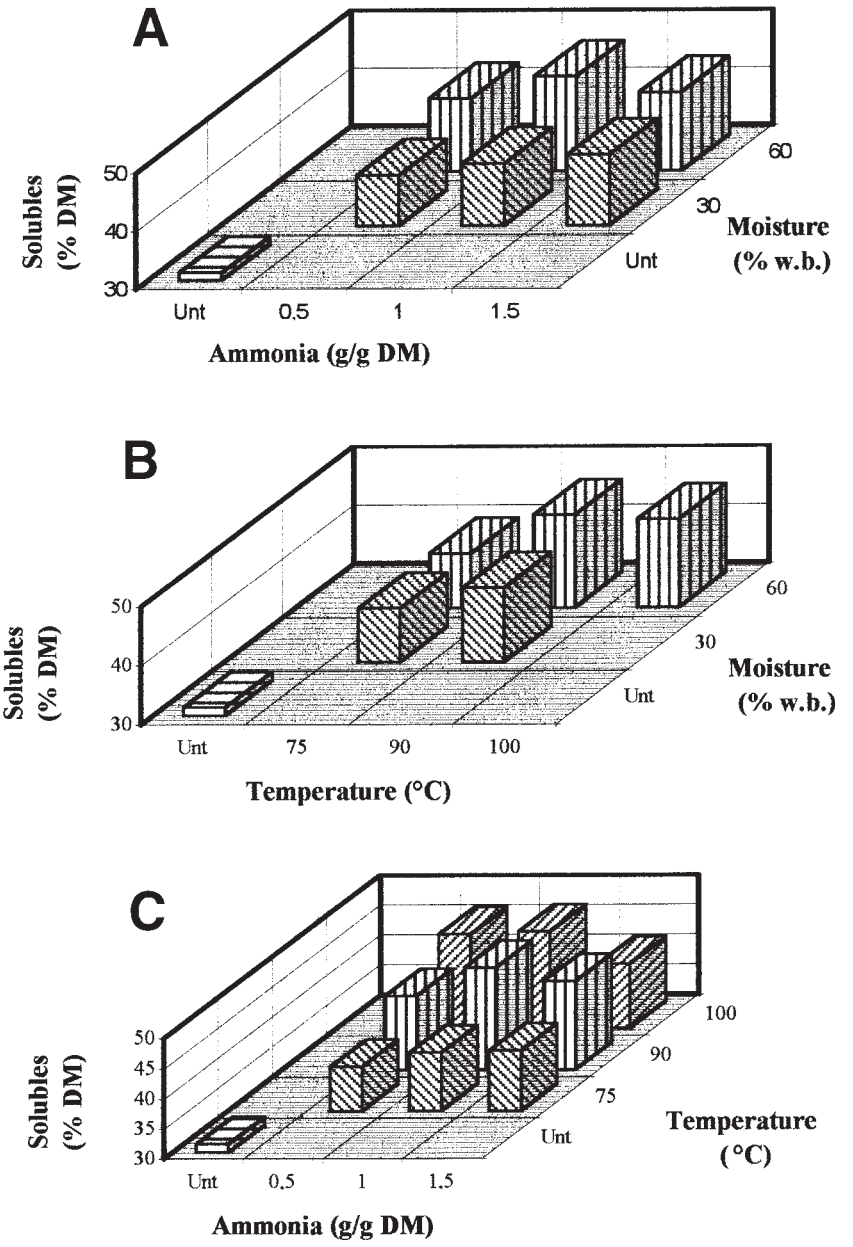

Fig. 2. Solubles content of untreated (Unt) and PDA-treated DEG, at several **(A)** *r-M* combinations, **(B)** *M-T* combinations, and **(C)** *r-T* combinations. SEM values: (A) 0.40; (B) 0.36; (C) 0.31.

The best treatment condition to increase solubles and decrease hemicellulose was 1.0-60%-90°C, while maintaining a similar cellulose content compared to untreated DEG ($p > 0.05$), as shown in Table 1. Contrary to AFEX *(26)*, *r* and *M* play a major role in the efficacy of the treatment. Temperature also plays a major role and must be controlled since high tempera-

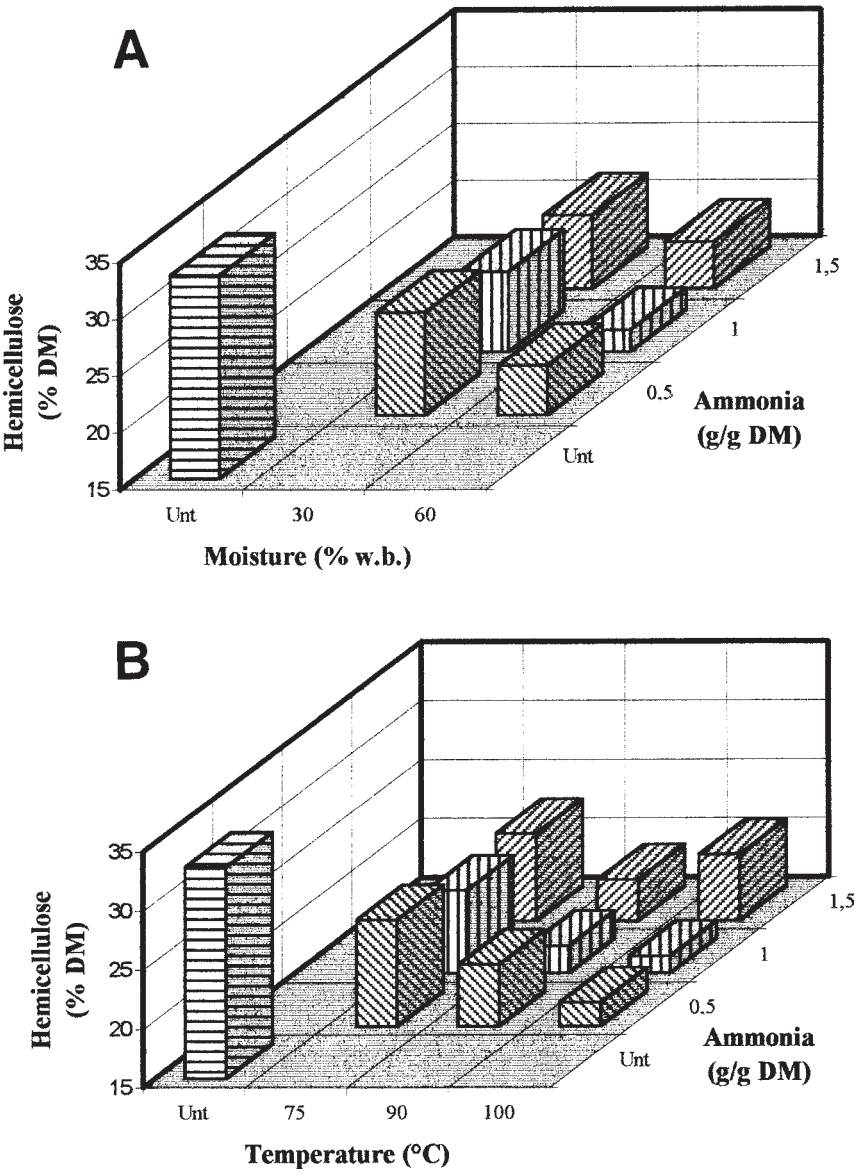

Fig. 3. Hemicellulose content of untreated (Unt) and PDA-treated DEG, at several **(A)** *M-r* and **(B)** *T-r* combinations. SEM values: (A) 0.38; (B) 0.47.

tures at high moistures and ammonia loadings may deteriorate the biomass. This has been confirmed by measuring ruminal digestibility of PDA-treated alfalfa *(13)*.

Effect on Sugar Yield

Initial sugars of the grass were glucose (12 mg/g of DM), fructose (8 mg/g of DM), and sucrose (6 mg/g of DM), totaling 26 mg/g of DM.

Table 3
Initial Sugars (mg/g DM) in Untreated and PDA-Treated DEG[a]

Forage	IS	S	G	F	Unk	RS
Untreated	26.0	6	12	8		45
Treated	14.5	9			5.5	8

[a]Xylose, galactose, and cellobiose were not detected in any hydrolysate. IS, sum of individual sugars (HPLC); S, sucrose; G, glucose; F, fructose; Unk, unknown disaccharide; RS, reducing sugars (DNS).

Neither xylose nor galactose, typical sugars of legumes, were detected. As the result of PDA treatments, glucose and fructose disappeared, whereas sucrose and probably another disaccharide (there was an unknown peak with a retention time of a disaccharide, 11.2 min) were formed. Sugar concentration based on reducing sugars was greater than that based on individual sugars (45 vs 26 mg/g of DM). This could be explained by the presence of a reducing sugar not accounted for by HPLC analysis, but there is no supporting evidence. In any event, sugar concentrations were extremely low. The results in Table 3 also show that hemicellulose was not hydrolyzed to sugars, as previously stated for ammonia reactor processes such as AFEX *(27)*, because xylose was not produced by the treatment. It is also possible to extract the sugars prior to PDA processing if they make a significant contribution to the total sugar yield. Vlasenko et al. *(28)* indicated that 10% higher yields were obtained with several pretreated forages after washing. If an evaporation process is to be used, then prewashing can be applied, provided that the amount of water is minimal to save drying energy.

Figure 4 shows the reducing sugars produced from untreated and PDA-treated DEG at 1, 2, and 5 IU/g of cellulase loading, respectively, using Spezyme CP and Novozym 188. Results show in general that PDA-treated samples have a much higher extent and initial rate of hydrolysis (Table 4) than untreated samples. Both extent and initial rate of hydrolysis tend to increase with enzyme loading.

Differences between PDA-treated and untreated DEG were marked, approx 2.1-fold. Enzyme loading had a relatively small effect in both untreated and PDA-treated DEG in terms of sugar production by 24 h. However, an enzyme loading of 2 IU/g produced significantly greater yields than 5 and 1 IU/g after 24 h ($p < 0.05$). A very high initial rate for 5 IU/g compared to 2 IU/g may have caused an early feedback product inhibition, which might explain the greater sugar yield with 2 IU/g after 24 h. Therefore, a level of 2 IU/g was chosen for the remainder of the treatments. Note that an enzyme loading of 1 IU/g appears feasible for DEG, which would reduce the cost of enzyme application. The extent of hydrolysis of treated DEG was about 76% of theoretical at 72 h, 64% of which was attained at 24 h. However, 24 h was chosen as the digestion time. Theoretical yield was 715 mg/g of dry forage, based on combined cellulose and hemicellulose contents of the forage (times the dehydration factor, 1.1).

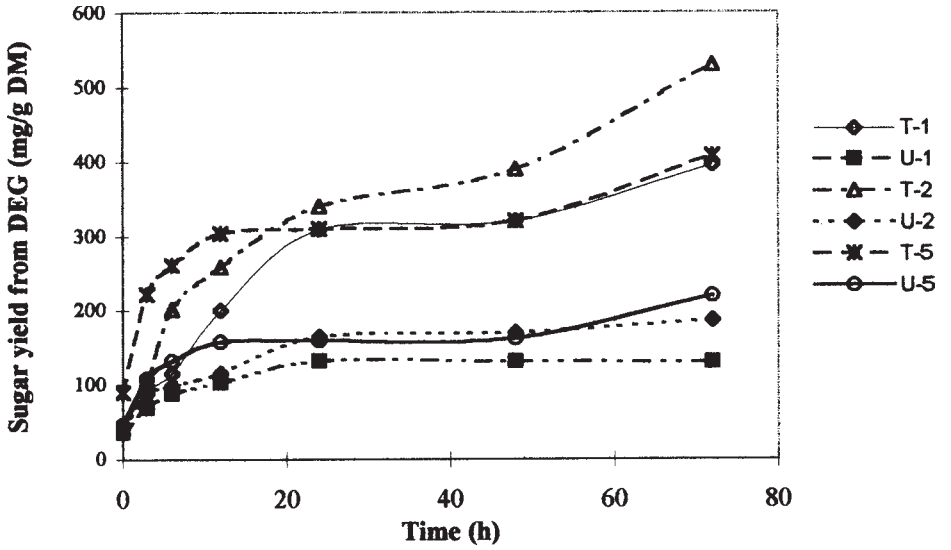

Fig. 4. Kinetics of enzymatic hydrolysis of untreated (U) and PDA-treated (T) DEG.

Table 4
Initial Digestion Rates
of Untreated and Treated DEG
by Spezyme CP and Novozym 188 at 1, 2,
and 5 IU/g Cellulase Loading

Enzymes (IU/g DM)	Rate (h^{-1})	
	Untreated	Treated
1	11.1	16.0
2	15.1	21.1
5	20.8	48.8

Figure 5 shows the individual sugars produced during the first 24 h of digestion with Spezyme CP and Novozym 188 with the optimal enzyme loading (the same experiment presented in Fig. 4). Based on theoretical yields from cellulose and hemicellulose of 352 and 363 mg/g of DM, respectively, untreated DEG had a cellulose conversion of 23% and a hemicellulose conversion of only 2.7%, indicating a high degree of resistance of hemicellulose to enzymatic hydrolysis. In addition, there was clearly a lag phase of several hours for hemicellulose hydrolysis. No other sugar besides glucose and xylose was produced, and the initial content of fructose remained unchanged. On the other hand, no fructose was present in treated DEG. Cellulose and hemicellulose conversions, reflecting mainly glucose and xylose production, increased to 52.6 and 14%, respectively, with the ammonia treatments, and corresponded to 2.3- and 5.2-fold increases for cellulose and hemicellulose conversions, respectively. In other words, in

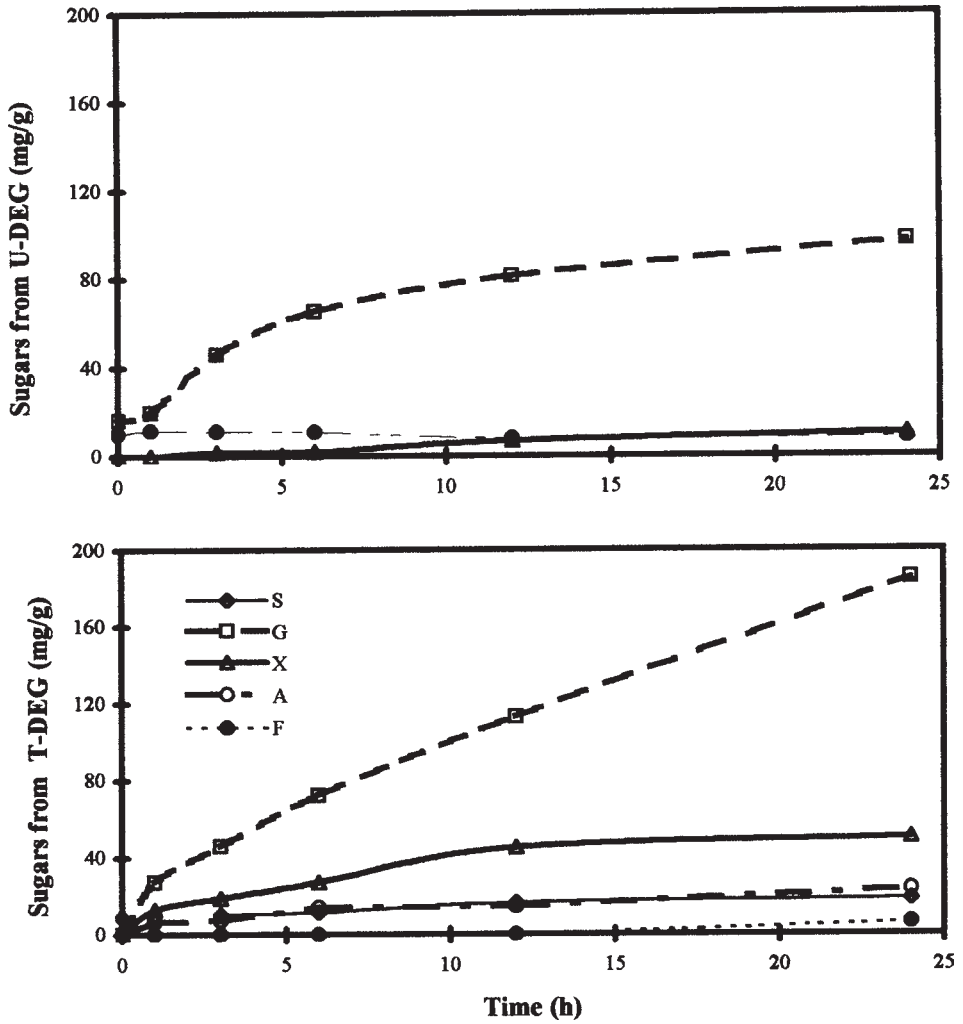

Fig. 5. Sugars released during enzymatic hydrolysis of untreated (U) and PDA-treated (T) DEG with Spezyme CP and Novozym 188. Cellulase activity: 2 IU/g. S, sucrose; G, glucose; X, xylose; A, arabinose; F, fructose.

DEG, the PDA treatment was more effective at increasing hemicellulose susceptibility to hydrolysis than cellulose. However, hemicellulose conversions were considerably lower than cellulose conversions; this could be owing to insufficient xylanase activity in Spezyme CP and Novozym 188 *(24,29)*.

Only in treated DEG was there a peak increasing with hydrolysis time at a retention time typical of disaccharides. This peak was either cellobiose nor maltose. However, could be xylobiose because Chou *(30)* found xylobiose as a major component of hydrolysis of lignocellulosic materials treated with ammonia at high temperatures. That peak amounted for 51 mg/g of DM of sugar.

Table 5
Sugar Yield and Glucose/(xylose + arabinose + galactose) Ratio Between Two-Enzyme[a] and Three-Enzyme[b] System Hydrolysis of Untreated (U) and PDA-Treated DEG

	Two-enzyme system[c]			Three-enzyme system[c]		
Forage	RS (mg/g)	IS (mg/g)	G/ (X + A + GAL)	RS (mg/g)	IS (mg/g)	G/ (X + A + GAL)
Untreated	164	116	9.7	188	151	4.3
Treated	340	241	3.3	397	398	1.5

[a]Spezyme CP and Novozym 188 (2 IU cellulase/g DM Spezyme CP).
[b]Spezyme CP, Novozym 188, and Multifect XL (2 IU cellulase/g DM Spezyme CP).
[c]RS, reducing sugars; IS, total individual sugars determined by HPLC; G, glucose; X, xylose; A, arabinose; GAL, galactose.

Total sugar yields based on HPLC analysis of individual sugars were approx 75% of the yields based on analysis of reducing sugars, which is in agreement with extensive previous reports *(31,32)*. This is probably owing to the presence of oligosaccharides, as well as a different response of the individual monomers to the DNS reagent. Reducing sugars may exhibit different ring and open-chain equilibria, which affects the stoichiometry of the redox reactions; that is, xylose exhibits approx 85% of the response displayed by glucose *(31,33)*.

To test the hypothesis of insufficient xylanase activity, an experiment was performed by adding Multifect XL as a source of xylanase on an equal international units/gram basis as cellulase from Spezyme CP, and also adding cellobiase at the same loading ratio as before. A level of 2 IU/g of cellulase was used. Table 5 indicates that by adding a source of xylanase, sugar yields greatly increased. The sugar ratio for the three-enzyme mixture was lower than for the two-enzyme mixture, confirming the selective action of Multifect XL on hemicellulose. For this reason, Multifect XL was included in the enzyme mixture to test the effect of PDA-processing conditions on sugar yield from forages. Increasing the enzyme loadings of Multifect XL did not prove to be of further use (results not shown).

PDA treatment dramatically increased ($p < 0.0001$) the susceptibility of the fibers to enzymatic hydrolysis measured as reducing sugars. Untreated DEG yielded 170 mg of reducing sugars/g, whereas the 1-60-90 treatment yielded 580 mg/g (Figure 6), a 3.7-fold increase and 81% of the theoretical yield. Treatment 1-60-100 yielded 83% sugar, but was not significantly different from treatment 1-60-90 ($p > 0.05$).

Main effects were significant among treatments ($p < 0.0001$). Figure 6A shows that $r = 1.5$ is detrimental when used in combination with 60% *M* and 90°C, confirming the same trend observed with solubles and hemicellulose data. Higher moisture increased sugar yields (Fig. 6B), and 90°C was a better temperature than 75°C ($p < 0.0001$) (Fig. 6C).

Table 6
Enzymatic Hydrolysis Yield (24 h)
of Glucose, Xylose, and Arabinose of Untreated and PDA-Treated DEG

Treatment (*r-M-T*)[a]	Glucose (mg/g DM)	Xylose (mg/g DM)	Arabinose (mg/g DM)
Untreated	95	10	4
05-60-90	258	77	16
1-60-90	272	99	17
1.5-60-90	299	66	14
1-30-75	254	34	11
1-60-75	260	57	13
1-30-90	251	50	10
1-60-100	299	66	16

[a]*r*, ammonia loading (g NH_3/g DM); *M*, moisture (% w.b.); *T*, temperature (°C).

The final concentration of sugars in the filtrate was approx 3% (w/v) in the best treatments. More important, this increase was achieved with low enzyme levels (2 IU/g) of cellulases and xylanases during a 24-h incubation. Much of the work done on hydrolysis of chemically treated materials has been done with enzyme dosages of 20–100 IU/g *(34–36)*. Ammonia treatments such as AFEX have produced sugar yields at 90% of theoretical on coastal Bermuda grass *(10)* and switchgrass *(1)* with 5 IU of cellulase/g using Cytolase 300 (Genencor). In our experiment, the dosage was reduced (compared to AFEX) from 5 to 2 IU/g, and a treatment time of 5 vs 20–30 min was used. It may be possible and economically advantageous to use 1 IU of cellulase/g, but it could require longer hydrolysis times. It has been possible to hydrolyze AFEX-treated corn fiber with 1 IU of cellulase/g *(37)*, producing a 2.1% sugar solution, which is approx 33% lower than the concentration obtained in the current study.

Recent reports (e.g., *see* ref. *24*) have indicated that corn fiber hemicellulose is only partly hydrolyzed even with high xylanase activity, which indicates that cellulose conversion is very high but that of hemicellulose is not. A lime treatment was recently applied to switchgrass attaining 80% of theoretical sugar yield *(32)*. This conversion required 5 FPU/g of cellulase, 5% solids, and 72 h of incubation. In addition, the lime treatment lasted 2 h at 100–120°C, which is extremely energy demanding.

The sugar yield HPLC data revealed some important facts (Table 6). Increasing *r* (1–1.5) and *T* (90–100°C) increased cellulose hydrolysis but decreased that of hemicellulose. The total amount of sugars was similar. The data also show that low temperature (75°C) and moisture (30%) caused a much smaller extent of hydrolysis, showing a greater reduction for hemicellulose than for cellulose, indicating again the positive effect of PDA on hemicellulose at optimal conditions.

The HPLC data undoubtedly suggest the presence of hemicellulose oligosaccharides. In PDA-treated DEG, cellulose conversion (based on glucose production) reached a top value of 85% but hemicellulose conversion

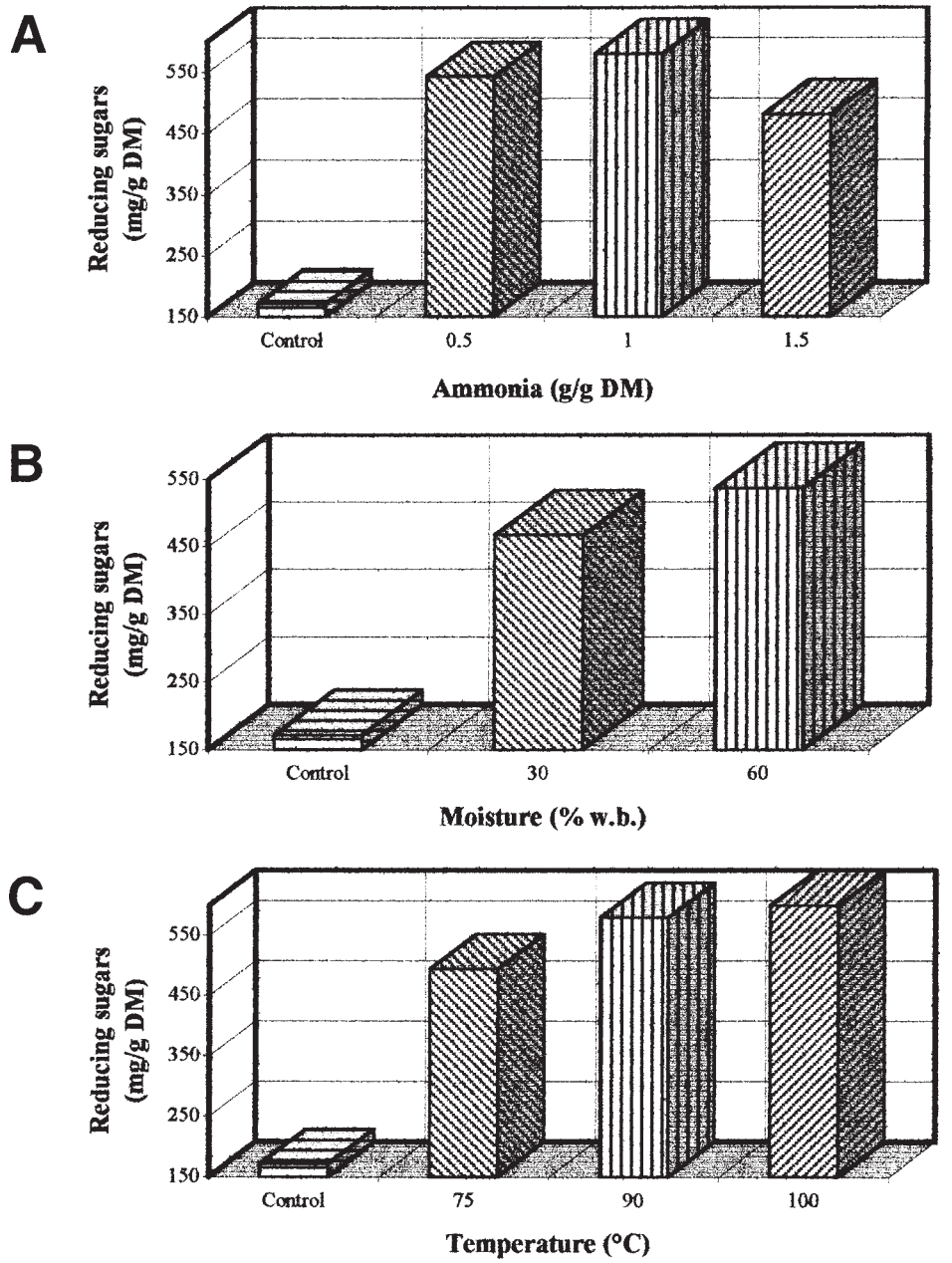

Fig. 6. Reducing sugars from enzymatic hydrolysis of untreated and PDA-treated DEG at selected **(A)** ammonia loadings (60% *M*, 90°C), **(B)** moisture contents (1 *r*); and **(C)** temperatures (1 *r*, 60% *M*). SEM values: (A) 6.9; (B) 5.7; (C) 6.9.

(based on xylose plus arabinase production) was only 33%, although both were considerably higher than in untreated DEG (27% and 3.9%, respectively). Therefore, cellulose and hemicellulose conversions increased 3.2- and 8.2-fold, respectively (using the three-enzyme system). However,

reducing sugar yield was 83%, indicating high degradation of both cellulose and hemicellulose. Since the enzyme mixture had sufficient cellobiase *(28)*, the result of cellulose hydrolysis was mainly glucose. On the other hand, hemicellulase action on lignocellulosics has been reported as limited mainly to oligosaccharides *(24)*. Walch et al. *(38)* reported a 60% conversion into xylose of xylan extracted from hydrothermally treated sugar cane bagasse, but they used a dosage of 667 IU/g of xylanase, which is 333-fold the dosage used in the present study. In addition, DEG is a forage with a very high hemicellulose content. It is important to note that cellulose hydrolysis is enhanced if hemicellulose is removed *(29)*. In our study, the PDA treatment initially removed about 50% of the hemicellulose, and, later, hydrolysis degraded the already solubilized hemicellulose plus that remaining in the solid fraction.

Conclusion

Ammonia loading, moisture, and temperature play a major role in PDA treatments. An increase in either one of these parameters tends to cause an increase in hemicellulose solubilization and an increase in sugar yields. However, at high r-M-T combinations, hemicellulose solubilization decreases as well as hemicellulose hydrolysis. On the other hand, cellulose hydrolysis still increased at the conditions studied. Ammonia treatment did not produce either xylose or arabinose from hemicellulose.

The production of high sugar yields (83% of theoretical yield), together with low enzyme levels (2 IU/g of DM) and short incubation times (24 h), allows DEG to be a potential feedstock to produce economical sugars for further conversion into ethanol, organic acids production, and for animal feeding. It seems possible to reduce the enzyme loading even more. In the specific case of hemicellulose, more efficient enzymes are needed to convert it to its monomers in a greater extent.

PDA treatments in this study were investigated at relatively mild temperatures with short reaction times (5 min). These results are encouraging and suggest the need for further research in the application of PDA treatments to many other agricultural materials currently available in Venezuela, such as sugar cane bagasse and corn stubble, as well as forages.

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