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Optimizing Ammonia Processing Conditions to Enhance Susceptibility of Legumes to Fiber Hydrolysis

Florigraze Rhizoma Peanut

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Abstract

A warm-season legume, Florigraze rhizoma peanut (FRP), was used as the source of fiber to produce sugars. FRP was subjected to several ammonia-processing conditions using temperature, biomass moisture content, and ammonia loading as process variables during a 5-min treatment. A cellulase loading of 2 FPU/g DM and 24 h incubation were used to produce the sugars. Total sugar yield was 3.34-fold higher in the optimal treatment (1.5 g ammonia/g DM–60%–90°C) compared to untreated and was 65.3% of theoretical. Cellulose and hemicellulose conversions increased from 30 and 15.5% in untreated FRP to 78 and 34% in treated FRP.

Index Entries: Ammonia; florigraze rhizoma peanut; enzymatic hydrolysis; sugars.

Introduction

Tropical countries, most of them underdeveloped, need to address the problem of insufficient feed and food sources. Venezuela, for example, has a great deficit of protein foodstuffs for nonruminants. Studies have shown that a great fraction of the protein can easily be recovered from treated materials following ammonia processing *(1–3)*. This can be extremely

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important in the case of legumes owing to their relatively high protein content. However, the fiber remaining after processing must have significant value, for instance, improved digestibility, to make the process profitable. This goal might be reached by finding ammonia-processing conditions that convert a great fraction of legume fibers into sugars, the main objective of this work. Ammonia processes have been successfully used as pretreatments to enhance sugar production in dwarf elephant grass *(4)*, rice straw *(5,6)*, and alfalfa (ALF) *(7)*. Cost of ammonia recovery will likely not be higher than $10 per ton of biomass treated and recovery could go up to 99% *(7)* making the process economically attractive. The tropical legume selected for this study was florigraze rhizoma peanut (FRP).

FRP (*Arachis glabrata* Bench) is a perennial high-quality legume adapted to well-drained soils in warm areas throughout the tropics and subtropics *(8)*. There is considerable interest in growing FRP through the extreme southern region of the United States, mainly Florida and the southern Gulf Coast *(9)*. Interest in FRP is based mainly on its drought tolerance once established and its nutritive value (energy) comparable to ALF (although it has a lower crude protein content) *(10)*. FRP is also being considered as an alternative forage in Central and South America. Unlike most tropical legumes, FRP is productive and persistent over a relatively wide range of grazing management practices *(11)* and can be grown successfully with various perennial grasses for several years *(12)*.

Materials and Methods

Ammonia Processing

A laboratory-scale batch ammonia reactor unit consisting of a 4-L reactor with appropriate support equipment was used to process 80-g samples (dry matter) as explained elsewhere *(7)*. FRP was grown near Beeville (TX) and received as hay (8% moisture, w.w.b). The hay was ground in a Wiley mill to 20 mesh and kept under refrigeration until used.

FRP processing was carried out at ammonia loadings of 0.5, 1, and 1.5 g ammonia/g dry matter (DM), moistures of 30 and 60% (w.w.b.), and temperatures of 75 and 90°C ($3 \times 2 \times 2$ factorial for ammonia loading, moisture, and temperature). Dwell time was 5 min. Untreated FRP was used as a control for all experiments. Ammonia treatments are labeled as r–M–T (ammonia loading–moisture–temperature) treatments throughout this article for a dwell time of 5 min.

Fiber Fractionation

Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined on triplicate samples to estimate cellulose, hemicellulose, and lignin by sequential fractionation using reagents and procedures recommended by Goering and Van Soest *(13)*. "Solubles" concentration (%) is calculated by subtracting NDF from 100, and refers to soluble cellular components (proteins, sugars, oligosaccharides, organic acids, lipids) in addition to pectic substances. Crude protein was analyzed as Kjeldahl nitrogen.

Determination of Optimal Enzyme Loading and Hydrolysis Time

The untreated and the ammonia-treated sample with the lowest amount of hemicellulose were subjected to enzymatic hydrolysis by cellulase and cellobiase *(7)*. Hydrolysis conditions and sample processing for reducing sugar determination were the same as those indicated for dwarf elephant grass *(4)*. 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 DNS method *(15)*. A high-pressure liquid chromatographic (HPLC) analysis was also carried out on the samples *(4)*. Optimal enzyme loading and hydrolysis time were chosen and used subsequently to test the effect of ammonia processing conditions on sugar production. Initial hydrolysis rates were estimated from the kinetic study.

Sugar Production at Different Treatment Conditions

Untreated and all treated samples were subjected to enzymatic hydrolysis at optimal cellulase loading and hydrolysis time, and reaction conditions as before, except for the addition of hemicellulase (Multifect XL Genencor, Inc., San Francisco, CA) at the same level as cellulase. Sugar production was measured as reducing sugars at zero time and at 24 h with the DNS method *(15)*. Sugar yield was also expressed as percentage of theoretical conversion taking into account both cellulose and hemicellulose content of untreated FRP. Sugars initially present in the samples (estimated after 5 h incubation with no enzymes added) and sugars present in the enzyme solutions were subtracted from total sugar determination to estimate net sugar yields. The results of sugar yield were analyzed using general linear methods (GLM) of the Statistical Analysis System (SAS) *(16)*. Sample variation was expressed as the standard error of the mean (SEM).

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 zero and 24 h hydrolysis were determined by HPLC analysis as explained elsewhere *(4)*. This analysis was carried out on the untreated and also on selected treated samples.

Results and Discussion

FRP used in this work had 48.3% NDF, 27.2% ADF, 6.2% ADL, and 13.7% CP, which corresponds to 51.7% solubles, 21.1% hemicellulose, 21.0% cellulose, and 6.2% lignin. These values compare well with those reported previously for hay made from young FRP, but with a lower ADF content *(10)*. As a result of ammonia treatments the solubles content of untreated FRP increased from 51.7% to 64.1%, a 24% increase for the best treatment, 1–60–90 (Table 1). Hemicellulose decreased from 21.1% to 9%, a 57.3% reduction. Cellulose contents of untreated samples and 1–60–90 treated

Table 1
Effects of Optimal Ammonia Treatment Conditions[a]
on Fiber Fractionation Values of Florigraze Rhizoma Peanut

Variable	Control value[b] (% DM)	Treatment value (% DM)	Net change (%)
Solubles	51.7	64.1	24.0
Hemicellulose	21.1	9.0	−57.3
Cellulose	21.0	21.4	1.9
Lignin	6.2	5.87	−5.3

[a]Optimal treatment condition (highest reduction on hemicellulose content) = 1−60−90 during 5 min.

[b]Values of untreated FRP were significantly different from ammonia-treated FRP ($p < 0.0001$), except for cellulose.

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

	Rate (h^{-1})	
Enzymes (IU/g DM)	Untreated	Treated
1	2.7	15.6
2	3.2	19.7
5	5.5	27.5

samples were similar. The NDF value of ammonia-treated FRP was reduced to 35.9%, which is typical of ALF *(17)*, a better forage legume. In addition, lignin was reduced by 11.6%. Solubles were likely produced by partial hydrolysis *(18)* and solubilization of hemicellulose by the alkaline treatment *(19)*. Reduction of hemicellulose content, although low in forages treated with simple ammoniation *(20)*, was again high in ammonia reactor processing *(4,7)*.

Figure 1 shows that all ammonia-treated FRP samples had much higher extent and rate of hydrolysis (Table 2) than untreated FRP. Differences between treated and untreated FRP were greater than those in alfalfa *(7)*, but differences among the legumes were much smaller than those for grasses such as dwarf elephant grass *(4)*. The theoretical yield is 464 mg sugars/g dry forage based on combined cellulose and hemicellulose contents of the forage (times the dehydration factor, 1.1). For this two-enzyme system, the sugar yield at 72 h was 60% of theoretical. Initial rates (2.7–5.5 h^{-1}) were similar to those for alfalfa *(7)*, but much smaller than those for dwarf elephant grass *(4)*. The initial rate for a cellulase loading of 5 IU/g was greater than for 2 IU/g, but, at 24 h, the extent of hydrolysis was similar

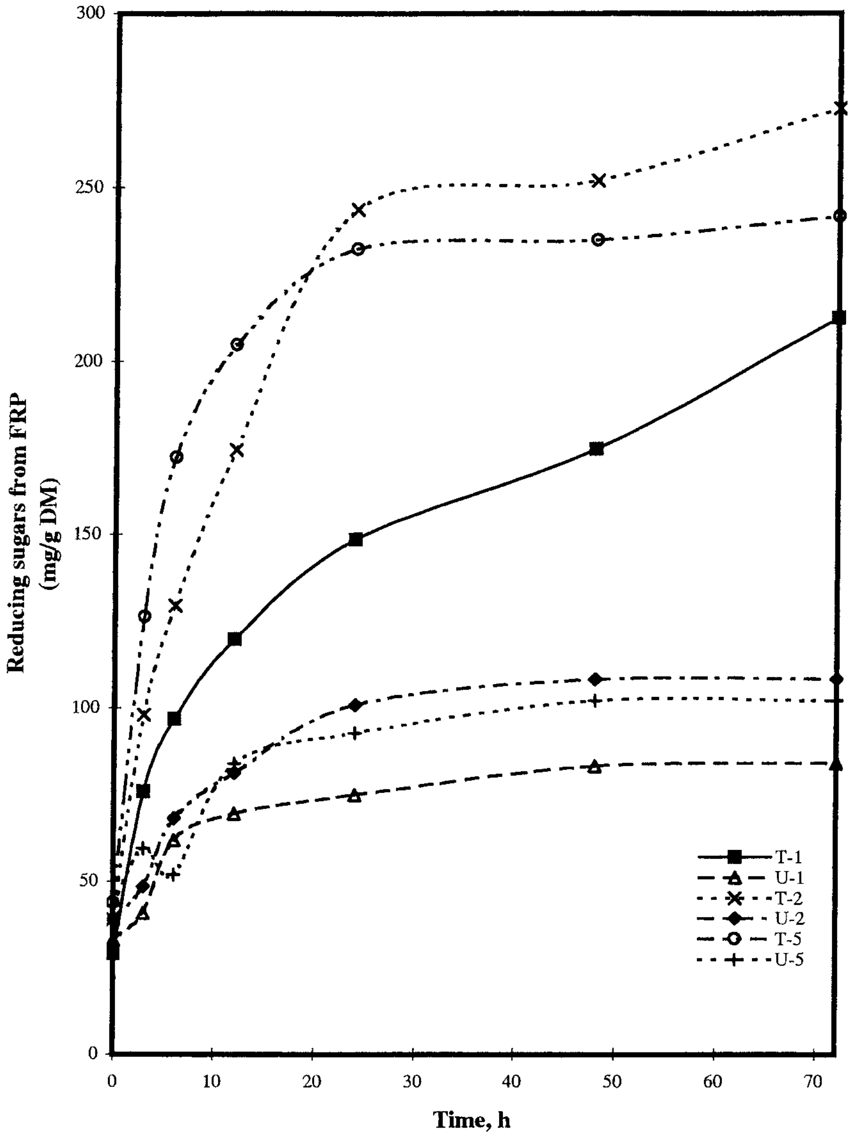

Fig. 1. Kinetics of enzymatic hydrolysis of florigraze rhizoma peanut. U-1, U-2, and U-5: untreated FRP at cellulase loadings of 1, 2, and 5 IU/g DM, respectively. T-1, T-2, and T-5: ammonia-treated FRP at cellulase loadings of 1, 2, and 5 IU/g DM, respectively. Enzyme mixture: Spezyme CP and Novozym 188.

for both enzyme loadings. Rates decreased sharply after that. However, since differences after 24 h were not significant ($p > 0.05$) and extent of hydrolysis at 24 h was 90% of that at 72 h, a 2 IU/g and a 24 h digestion time were selected for hydrolysis of the rest of the treated samples.

Table 3 shows the initial sugar contents of untreated and ammonia-treated FRP. Sugars initially present were glucose, xylose, and fructose.

Table 3
Initial Sugars (mg/g DM) in Untreated and Ammonia-Treated FRP[d]

Sample	IS[a]	Sucrose	Glucose	Xylose	Fructose	Unk[b]	RS[c]
Untreated	62	—	24	22	16	—	50
Treated	48	10	7	23	—	8	12

[a]Sum of individual sugars (HPLC).
[b]Unknown disaccharide.
[c]Reducing sugars (DNS).
[d]Galactose was not detected.
[e]—: not detected.

FRP had a high sugar content, similar to alfalfa, and this is characteristic of legumes (6–10% sugars). As the result of ammonia treatments, glucose and galactose either decreased or disappeared, while sucrose and probably another disaccharide (there was an unknown peak with a retention time typical of a disaccharide) were formed. This effect was also observed in dwarf elephant grass and alfalfa *(4,7)*. When reducing sugars are compared between untreated and treated samples, it appears that sugar content decreases considerably with treatment. HPLC data, nevertheless, show a much smaller reduction in individual sugars (22.6%). Smaller reducing sugar contents in ammonia-treated samples might be explained partly by the presence of nonreducing sugars in the treated forages (i.e., sucrose, including the unknown disaccharide) and underestimation of pentoses with the DNS method *(21)*. Xylose concentrations remained constant in the legume indicating that it did not react during ammoniation. Hemicellulose was not hydrolyzed to monomers but likely to oligomers, which is in agreement with other papers on ammonia processing *(4,7,22)*.

Figure 2A shows that only glucose increased steadily during the whole 24 h period in untreated FRP, whereas arabinose, fructose, and xylose concentrations stopped increasing at 6 h, which may be due to very low xylanase activity in Spezyme CP *(23)*. On the other hand, glucose and xylose were produced continuously throughout the time in treated FRP (Fig. 2B).

Figure 3 shows FRP hydrolysis data based on reducing sugars production. An ammonia loading of 1 g/g DM (Fig. 3A) produced the best results, being significantly higher than 0.5 and 1.5 ammonia loadings ($p < 0.05$). Figure 3B shows similar sugar yields for 30 and 60% moisture, but hemicellulose solubilization was usually greater at higher moisture (results not shown). Ammonia treatment at both moisture levels significantly increased sugar yield compared to untreated FRP ($p < 0.05$). The data failed to detect differences between 75 and 90°C (Fig. 3C). As with alfalfa, FRP might be used in an integrated fiber–protein–sugar forage processing industry. The possibility of using 75°C for sugar production appears promising for protein production in terms of protein quality and, in general, to save energy

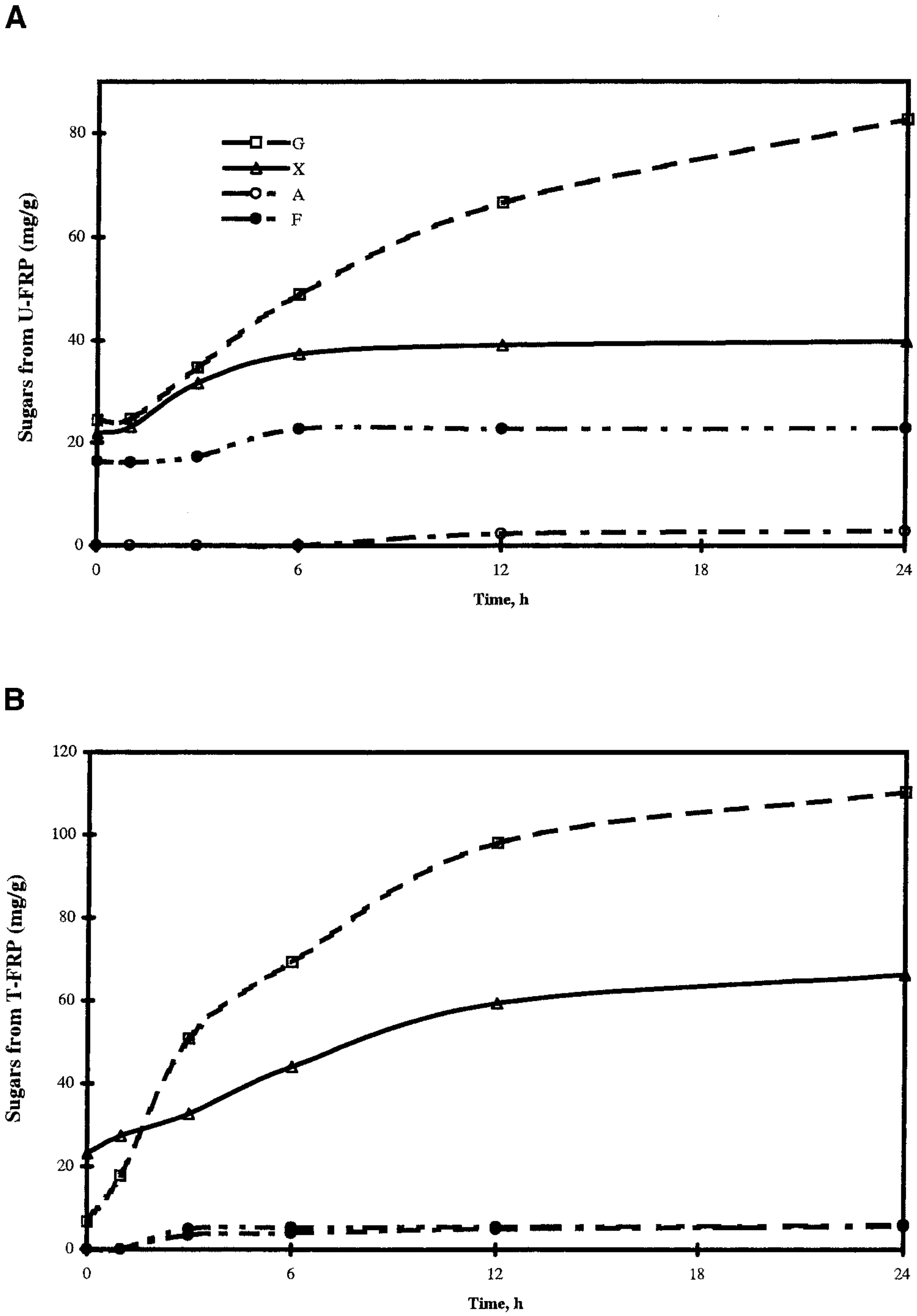

Fig. 2. Sugars released during enzymatic hydrolysis of untreated (U) and ammonia-treated (T) FRP with Spezyme CP and Novozym 188. Cellulase activity: 2 IU/g DM. G: glucose, X: xylose, A: arabinose, F: fructose.

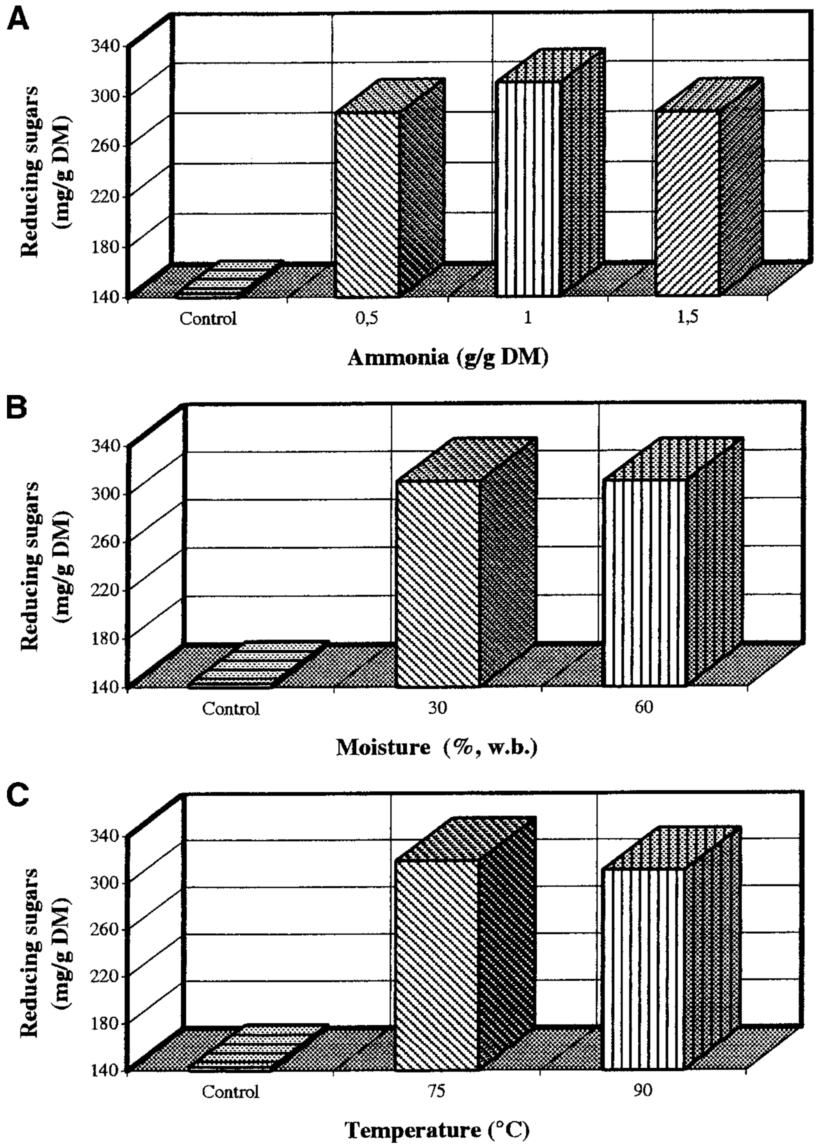

Fig. 3. Reducing sugars from enzymatic hydrolysis of untreated and ammonia-treated FRP at selected **(A)** ammonia loadings, **(B)** biomass moisture contents, and **(C)** temperatures. SEM values: (A) 6.6, (B) 6.3, (C) 6.3.

consumption. Protein damage due to ammonia treatments may only occur under more severe conditions, such as higher temperatures and with treatment times of 30 min or longer *(24)*. On the other hand, insufficiently high temperatures in ammonia-treatment processes greatly decrease efficiency of protein extraction and lower sugar yields, thereby requiring higher enzyme loadings *(2)*.

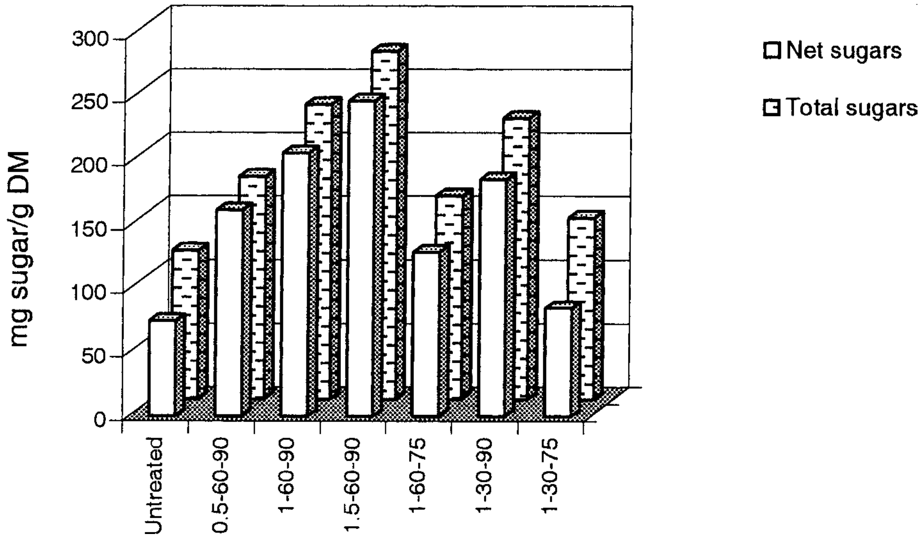

Fig. 4. Net and total sugars produced by enzymatic hydrolysis (24 h) of FRP. Enzyme mixture: Spezyme CP, Novozym 188 and Multifect XL. Total sugar includes initial sugar.

Figures 4 and 5 show HPLC data for selected treatments. Figure 4 shows both net sugars (produced by enzymatic hydrolysis) and total sugars (including sugars initially present in the samples). There was considerable variation in sugar yield with treatment conditions. Sugar yield increased with ammonia loading as observed in alfalfa *(7)*. However, 1.5 g ammonia / g DM was too much ammonia for dwarf elephant grass at equal moisture and temperature conditions *(4)*, indicating that processing conditions are apparently specific for each material. It appears that temperature increases sugar yield to a greater extent than does increasing moisture content. The 1.5–60–90 treatment produced the greatest sugar yield (based on the individual sugar data), which was 20% greater than the yield for the 1–60–90 treatment. Reducing sugar data did not show such a difference between these two treatments. The lowest yields were associated with one low processing condition, 0.5 *r*, 30% *M*, or 75°C. Total sugar yields in the two best treatments were about 10–12% higher than their corresponding net sugar yields. This proportion was much higher in the untreated samples and 75°C treatments, suggesting that a fraction of the sugar initially present in the materials might be lost at the most severe ammonia treatment conditions.

Figure 5 indicates that the 1.5–60–90 treatment had the highest net sugar yield because it had the greatest glucose yield. The treatments 1–60–90 and 0.5–60–90 produced similar glucose yields, but as xylose production by the 0.5–60–90 treatment was small and no arabinose was detected in the hydrolysate, net sugar yield for the 1–60–90 was greater than that for the

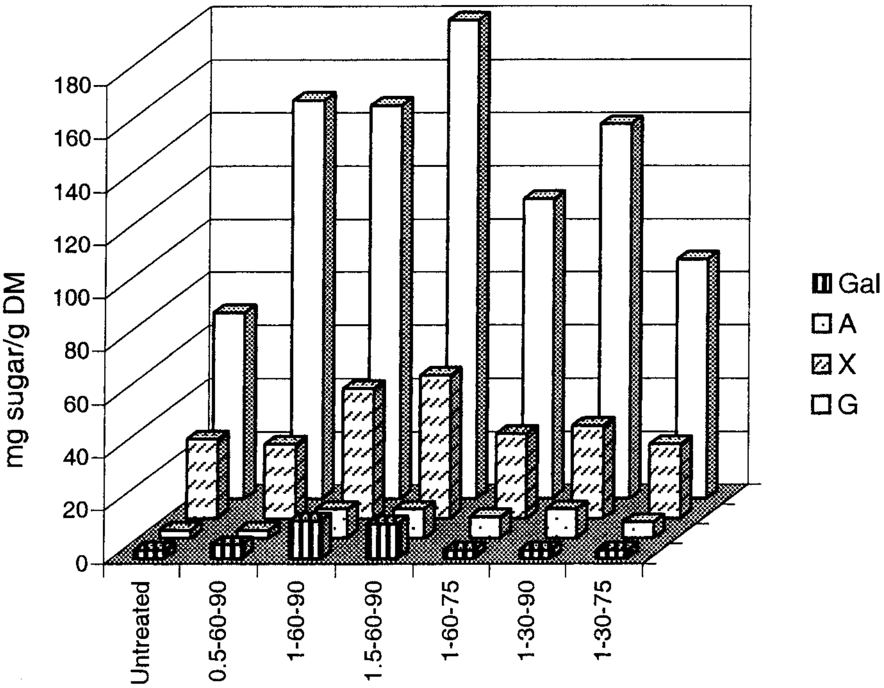

Fig. 5. Glucose (G), xylose (X), arabinose (A), and galactose (Gal) produced by enzymatic hydrolysis (24 h) of FRP. Enzyme mixture: Spezyme CP, Novozym 188 and Multifect XL.

0.5–60–90 treatment (Fig. 4). Galactose was only released in under 60%–90°C treatment combinations, and it was significantly higher in the 1 and 1.5 ammonia loading treatments than in the 0.5 g ammonia/g DM treatment ($P < 0.05$). Cellulose and hemicellulose conversions were 78 and 34%, respectively, much higher than in untreated FRP (30 and 15.5%, respectively). The best yield was 65.3% of theoretical (vs 19.6% in untreated), which is a little smaller than for alfalfa, 76% *(7)*, and much smaller than for grasses such as dwarf elephant grass, 83% *(4)* and coastal bermuda grass, 90% *(1)*, although similar to rice straw *(5)*. Lignin reduction in treated FRP reached 11.6% (Table 1), whereas alfalfa and dwarf elephant grass had about 20 and 38% reductions in lignin content, respectively *(4,7)*, which might explain these results.

It is important to point out that these data (Figs. 4 and 5) were produced with a three-enzyme system (cellulases, cellobiase, and xylanases), whereas data in Fig. 3 were produced with a two enzyme system (cellulases and cellobiase). When Multifect XL was included in the enzyme mixture as a source of xylanases, glucose production increased and xylose production increased even more. This might be due to presence of cellulase activity in Multifect XL, and more likely, that in legumes hydrolysis of hemicelluloses enhances cellulose hydrolysis. In addition, solubilization of hemicellulose from the complex matrix that links it to lignin and that hinders cellulose digestion, may increase susceptibility of the fibers to digestion, and particularly for ammonia treatments, may increase the efficacy of the response, rendering fiber more exposed for degradation.

Conclusions

Initial glucose and fructose concentrations decreased or disappeared with the ammonia treatment. Xylose concentration remained constant. The ammonia treatment did not hydrolyse hemicellulose to monomers.

Ammonia treatments greatly increased susceptibility of FRP to fiber hydrolysis, increasing sugar yield 3.3-fold compared to untreated FRP using cellulase and hemicellulase loadings of 2 IU/g DM and 24 h hydrolysis time. This suggests great potential to produce sugars from ammonia-treated FRP for either animal feeding or fermentation uses. The ammonia treatment increased the extent of hydrolysis of both cellulose and hemicellulose. Conversion of cellulose was relatively more enhanced than hemicellulose conversion with treatment. Ammonia loading and temperature appeared as the main factors affecting sugar yields. Hemicellulose solubilization enhances both cellulose and hemicellulose hydrolysis. The best processing condition was 1.5 g ammonia/g DM–60% moisture–90°C–5 min for FRP.

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