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

Alfalfa

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Abstract

An ammonia process was applied at several ammonia loadings, moisture contents, temperatures, and dwell times. A cellulase loading of 5 FPU/g dry matter and a 24 h incubation time were used to produce the sugars, which were measured as reducing sugars and by HPLC. Optimal processing conditions caused a 76% of theoretical yield (2.9-fold above untreated). Cellulose and hemicellulose conversions were 68 and 85% (vs 38 and 34% in untreated, respectively). The short hydrolysis time and relatively low enzyme loading suggests great potential to produce sugars from alfalfa.

Index Entries: Ammonia; alfalfa; enzymatic hydrolysis; sugars.

Introduction

Several physicochemical pretreatments are currently being tested to increase the value of forages. Most of them are not economically feasible due mainly to high cost, poor chemical recovery, degradation of the biomass, and large energy requirements. On the other hand, ammonia processes use a volatile reagent (ammonia) that is easily recovered, and the processing conditions are mild, which minimizes biomass degradation. In this process, the substrate is soaked with high-pressure (~300 psi) liquid ammo-

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nia at moderate temperatures (70–90°C) for about 2–5 min, causing cellulose to decrystallize and solubilizing part of the hemicellulose *(1,2)*. Then, the pressure is instantaneously released, causing the ammonia to flash violently disrupting the fibrous structure. The combined chemical effects (cellulose decrystallization and hemicellulose and lignin solubilization) and physical effects (increase in accessible surface area) markedly improve the susceptibility of lignocellulose to enzymatic hydrolysis.

Alfalfa is a very important forage in the United States. It has a higher protein content than grasses. When alfalfa leaves are sun-dried, the availability of the fibers is reduced as measured by its ruminal *in situ* dry matter digestibility, about 60%, which is considered low compared to high energy feedstuffs like grains (>80%). Structural limitations due to high lignin content and close association of lignin and hemicellulose (mainly ether and ester linkages) are responsible for this behavior *(3)*. On the other hand, several attempts to produce leaf protein from untreated alfalfa have failed due to poor recovery of protein in addition to the low value of the remaining fibers. Ammonia processing could both increase the value of the fibers as a feedstuff for sugars production and enhance protein extraction from alfalfa. Sugars could be also used as a source of energy for nonruminants. This work covers a wide range of processing conditions to identify those that produce fibers with a high susceptibility to enzymatic hydrolysis.

Materials and Methods

Ammonia Processing

A laboratory-scale batch ammonia reactor unit consisting of a 4-L reactor with appropriate support equipment was used for the treatment of alfalfa (*Medicago sativa*). Alfalfa (ALF) hay (18% moisture, w.w.b.) was obtained from the dairy (Texas A&M University), ground in a Wiley mill to 20 mesh, and kept under refrigeration until used. Water (adjusted to experimental level) and liquid anhydrous ammonia were added to 80-g samples (dry matter) and the temperature was rapidly raised to the experimental value. After the treatment time, pressure was suddenly released and the treated samples collected and allowed to air-dry overnight. Moisture contents are expressed in wet weight percent basis.

The first experiment was carried out to select an appropriate dwell time (t) for the ammonia treatment. Dwell times of 1, 5, and 10 min with an ammonia loading of 2 g ammonia/g dry matter (DM) and 30% moisture were tested at 85°C. The second experiment included three temperatures (T), 70, 85, and 100°C with an ammonia loading of 2 g/g DM at 30% moisture. The third experiment tested ammonia loadings of 0.5, 1, 1.5, 2, and 3 g ammonia/g DM at 30% and 60% moisture at 85°C. Dwell time for experiments 2 and 3 was 5 min. Untreated ALF was used as a control for all experiments.

Fiber Fractionation Analysis

Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined in triplicate to estimate cellulose, hemicellulose, and lignin *(4)*. Crude protein was estimated by Kjeldahl.

Determination of Optimal Enzyme Loading and Hydrolysis Time

Untreated and the ammonia-treated samples with the lowest amount of hemicellulose were subjected to enzymatic hydrolysis by cellulase (Spezyme CP, Genencor International Inc., 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 biomass during 72 h digestion. Cellobiase loading was kept at a 5.68 CBU/IU (cellobiase/cellulase) ratio. Cellulase activities were confirmed by the filter paper method *(5)*. Spezyme CP had 115.6 IFPU/mL. These data were used with cellobiase and xylanase activity information from the manufacturers to set the enzyme dosages. Hydrolysis was carried out as explained elsewhere *(2)*. 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 *(6)*. From this experiment, based on reducing sugar production, optimal enzyme loading and hydrolysis time were chosen to test the effect of 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 *(6)*. Sugar yield was also expressed as percentage of theoretical conversion taking into account both cellulose and hemicellulose content of untreated ALF. Sugars initially present in the enzyme solutions (3–6 mg/g DM) and sugars initially present in the substrates (determined after 5 h incubation to release the sugars) were subtracted from 24 h yields to estimate net sugar yields. The results of sugar yield were analyzed using general linear methods (GLM) of the Statistical Analysis System (SAS) *(7)*. An analysis of variance was applied to each of the studies and significance of the ammonia-treatment compared to untreated ALF, differences among ammonia treatments, effects of main variables (ammonia loading, moisture content, and temperature) and two- and three-way interactions were investigated. Sample variation was expressed as the standard error of the mean (SEM).

Degrees of cellulose and hemicellulose conversions were estimated based on the concentrations of glucose, and pentoses plus galactose and

Table 1
Effects of Optimal Ammonia Treatment Condition[a] on Fiber Fractionation Values of Alfalfa

Variable	Control value[b] (% DM)	Treatment value (% DM)	Net change (%)
Solubles	62.9	71.8	14.2
Hemicellulose	11.5	5.3	–53.5
Cellulose	19.7	16.5	–16.3
Lignin	5.9	4.74	–19.7

[a]Optimal treatment condition (highest reduction on hemicellulose content)= 2.0–30–85 during 5 min.
[b]Values of untreated ALF were significantly different from ammonia-treated ALF ($p < 0.0001$), except for cellulose.

mannose, in the hydrolysate, respectively. Sugar profiles for zero and 24 h hydrolysis were determined by high-pressure liquid chromatography (HPLC) as explained elsewhere *(2)*. Standard sugars were from Sigma (St. Louis, MO). This analysis was carried out in the untreated and in selected treated samples.

Results and Discussion

ALF used in this work (untreated) had 37.1% NDF, 25.6% ADF, 6.3% ADL, and 22.1% CP, or a 62.9% solubles, 11.5% hemicellulose, 19.7% cellulose, and 5.9% lignin. The sample with the lowest hemicellulose concentration (5.3% equivalent to a 53.5% reduction) was that treated at 2.0 g ammonia/g DM–30% moisture–85°C–5 min dwell time as processing conditions. This sample was used to investigate the optimal enzyme loading and hydrolysis time. This sample had the highest ruminal digestibility *(8)*, likely due to an increase in solubles caused by hemicellulose solubilization (53.5%), which is related to an increase in enzymatic susceptibility (Table 1). When dwarf elephant grass was treated with ammonia, the processing conditions that yielded the lowest hemicellulose content in the treated material also produced the highest sugar yield *(2)*.

Figure 1 shows the reducing sugars produced from untreated and ammonia-treated ALF at 1, 2, and 5 IU/g cellulase loading, respectively, using Spezyme CP and Novozym 188. Ammonia-treated samples had a much higher extent and initial rate of hydrolysis (Table 2) than untreated samples. There was a clear difference in performance between 5, 2, and 1 IU/g with 5 IU/g attaining approx 88% of the theoretical sugar yield at 72 h digestion; however, 77% yield can be attained by 24 h (which is nearly 90% of the 72 h yield). The theoretical yield is 342 mg/g dry forage based on the combined cellulose and hemicellulose contents of the forage (times the dehydration factor, 1.1). A 1 IU/g cellulase loading in treated ALF was sufficient to match the performance of a 5 IU/g loading in untreated ALF.

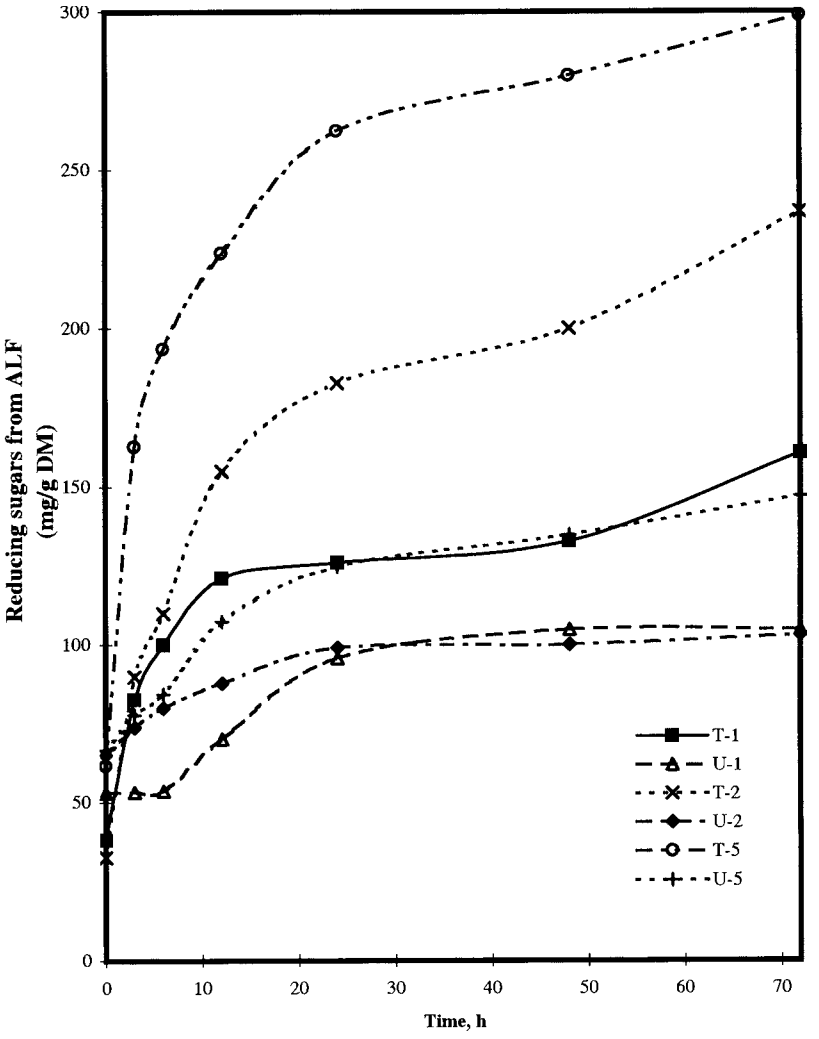

Fig. 1. Kinetics of enzymatic hydrolysis of alfalfa. U-1, U-2, and U-5: untreated ALF at cellulase loadings of 1, 2, and 5 IU/g DM, respectively. T-1, T-2, and T-5: ammonia-treated ALF at cellulase loadings of 1, 2, and 5 IU/g DM, respectively. Enzyme mixture: Spezyme CP and Novozym 188.

The difference in extent of hydrolysis was very marked between 5 IU/g vs 2 and 1 IU/g. Sugar yield for 5 IU/g at 24 h was about 1.5-fold that of 2 IU/g, 2-fold that of 1 IU/g, and 2.1-fold that of the best untreated result. Initial hydrolysis rates for untreated alfalfa were very low (0.1–3.7 h^{-1}) and considerably increased with the ammonia treatment (14.9-33.6 h^{-1}). The initial rates also increased with enzyme loading. A 5 IU/g enzyme loading was needed to make a substantial increase in hydrolysis for untreated samples, and was also the enzyme loading with the greatest yield in treated ALF.

Table 2
Initial Digestion Rates of Untreated and Ammonia-Treated Alfalfa 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	0.1	14.9
2	3.0	19.1
5	3.7	33.6

Table 3
Initial Sugars (mg/g DM) in Untreated and Ammonia-Treated Alfalfa

Sample	IS[a]	Sucrose	Glucose	Xylose	Galactose	Fructose	Unk[b]	RS[c]
Untreated	70	—[d]	23	18	11	18	—	64
Treated	65	26	10	21	—	—	8	18

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

At 24 h, the rate of hydrolysis decreased sharply; therefore, a 24 h digestion time and an enzyme loading of 5 IU/g were selected to test the rest of ammonia-treated ALF samples. A greater enzyme loading might produce greater yields, but at higher probable cost.

Table 3 shows the initial sugar contents of untreated and ammonia-treated alfalfa. Sugars initially present were glucose, xylose, fructose, and galactose. Contrary to Ben-Ghedalia and Miron *(9)*, mannose and arabinose were not detected in the water-soluble material from ALF, whereas xylose and fructose were detected representing 25.7% each of the sugar content. Lechtenberg et al. *(10)* reported fructose as one of the most abundant sugars in ALF solubles. Sucrose was not detected. The total neutral sugars present in ALF solubles (70 mg/g DM) is in agreement with the literature *(10)*. However, it is difficult to make comparisons because sugar levels depend on many factors, including maturity, height, time of cutting, and environment. 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. Smaller reducing sugar contents in ammonia-treated samples could be explained partly by the presence of nonreducing sugars in the treated forages (i.e., sucrose) and underestimation of pentoses with the DNS method *(11)*. Xylose concentrations remained fairly constant in the legume after treatment; therefore, there is no evidence that hemicellulose was hydrolyzed during ammoniation. On the other hand, it appears that galactose participated in condensation reactions because it was lost

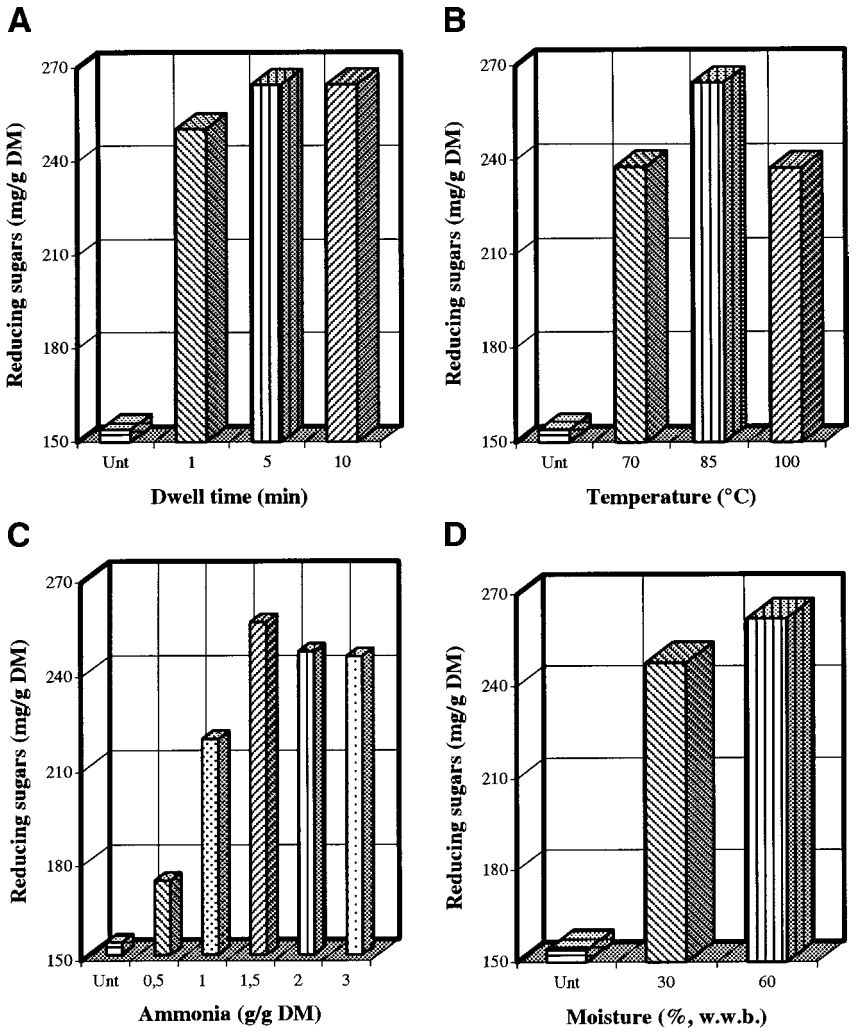

Fig. 2. Reducing sugars from enzymatic hydrolysis of untreated and ammonia-treated ALF at selected **(A)** dwell times, **(B)** temperatures, **(C)** ammonia loadings, and **(D)** biomass moisture contents. SEM values: (A) 10.7, (B) 9.4, (C) 9.5, (D) 7.5.

with treatment. One unknown peak (Table 3) could be a product of such condensation. Since fructose and almost half of glucose disappeared whereas sucrose appeared in the ammonia treated sample, it appears that ammoniation catalyzed sucrose formation.

Figure 2 shows the effects of dwell time, ammonia loading, moisture, and temperature on the reducing sugar yield. There were no significant differences ($p < 0.05$) among treatment dwell times (Fig. 2A); however, the 5-min and 10-min sugar yields were 6% higher than those for 1 min ($p < 0.10$). This difference was much less than the difference found in ruminal digestibility of treated alfalfa *(8)*, suggesting that at least for sugar production a 1 min treatment might be feasible. As in ruminal digestibility

data, Fig. 2B indicates 85°C as a better temperature than 70 and 100°C ($p < 0.05$). The detrimental effect at 100°C could be due to condensation reactions. Ammonia loading had a highly significant effect ($p<0.001$). Figure 2C shows 1.5 g ammonia/g DM as the best, followed by 2 and 3, although not significantly different than 1.5 ($p > 0.05$). Digestibility data showed both 1.5 and 2 as appropriate ammonia loadings to achieve high digestibility, with an ammonia loading of 3 being somewhat detrimental. Although all ammonia-treatment conditions produced significantly higher sugar yields than untreated ($p < 0.002$), ammonia loadings of 0.5 and 1.0 produced relatively lower sugar yields. No significant differences ($p > 0.05$) were found between the different moisture levels (Fig. 2D) when all the data were used due to the high variation between the untreated ALF replicates. However, most of the 60% moisture treatments produced greater sugar yields than the 30% (up to 13% higher). On the other hand, ruminal digestibility data clearly indicate that 60% was a better moisture level for most of the treatments. A very important ammonia × moisture interaction was found ($p < 0.05$) in the sugar yield data (Fig. 3). For a 1.5 ammonia loading, a 60% moisture was more effective, whereas for an ammonia loading of 2, a lower moisture was required. This effect was also suggested by digestibility data *(8)*. Such a result would be expected if ammonia solubilization in water played an important role in the effect, since 2 g ammonia/g DM–60% moisture, means a greater effect of ammonia loading, but at this level the material becomes less reactive likely due to condensation reactions. The best treatments were 1.5–60–85 and 2–30–85 for 5 min achieving a 76% of theoretical conversion, which was very high, considering that ALF fibers are regarded as very recalcitrant to degradation *(3)*. Ammonia treatments increased the sugar yield 2.9 fold (based on reducing sugars) above that of untreated ALF (76.4 vs 26.4%). Individual sugars data showed that cellulose and hemicellulose conversions increased from 38 and 34% in the untreated sample to 68 and 85% at the optimal treatment condition, respectively (1.8- and 2.5-fold, respectively). These results show that the ammonia treatment was apparently more effective on hemicellulose than on cellulose. The observed effect could be related to solubilization of a great fraction of the hemicellulose. Hydrolysis of ALF might be performed as part of an integrated forage refining industry in which ALF would be ammonia-processed, protein would be extracted, and highly digestible fibers used for ruminants or converted to sugars. This appears feasible because ALF protein is an excellent protein source for both ruminant and nonruminant animals *(12)*, and it has recently been shown that ammonia reactor processing greatly enhances protein extraction from forages *(13–15)*. In terms of economics, a 24 h digestion time with 5 IU/g enzyme loading is probably in the economic range, particularly if some enzyme recycling can be applied *(16)*. The feasibility further increases if enzymes can be produced *in situ (17,18)*.

Additional information was provided by the HPLC data for some selected treatments as shown in Figs. 4 and 5. The treatments last 5 min

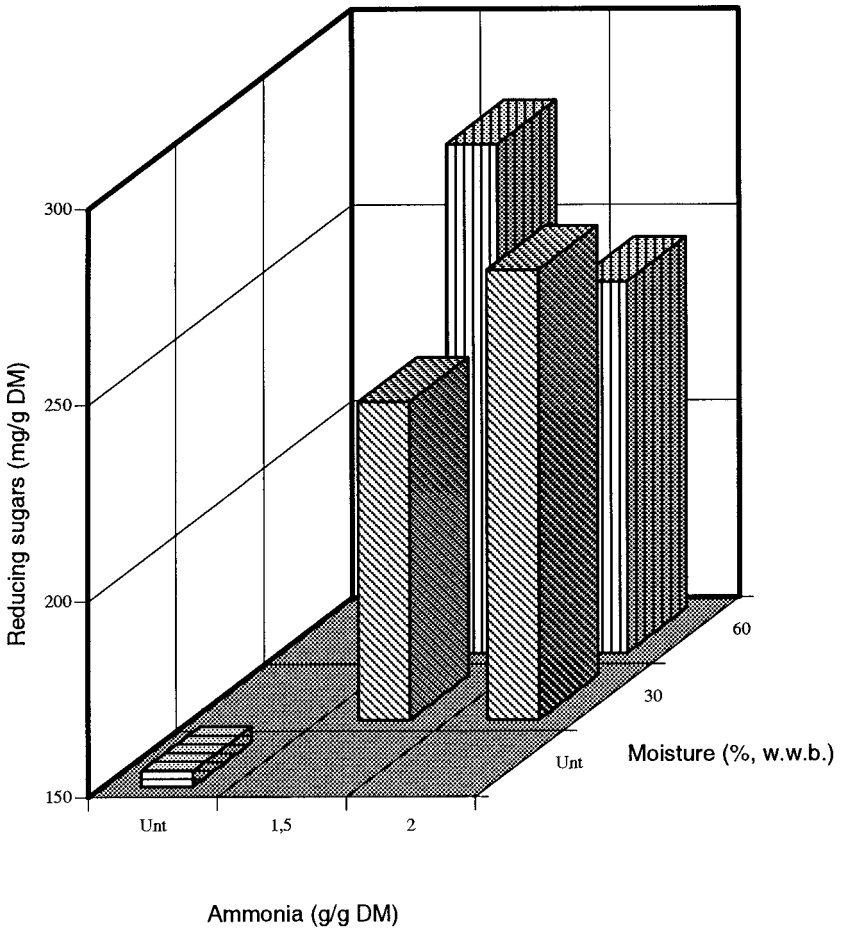

Fig. 3. Reducing sugars from enzymatic hydrolysis of untreated and ammonia-treated (85°C) ALF at selected moisture–ammonia loading combinations. SEM= 7.5.

except for the 2–30–85–1 and 2–30–85–10 treatments, which last 1 and 10 min, respectively. There was a marked increase in the production of glucose, xylose, and arabinose with increasing ammonia (Fig. 4). This was also true for mannose between 0.5 and 2 g ammonia/g DM; at 3 g ammonia/g DM mannose greatly decreased showing a deleterious effect, also shown in ruminal digestibility data *(8)*. Galactose also increased, but slightly (from 8 in untreated to 11 mg/g DM at 2 and 3 g ammonia/g DM). Cellobiose was not detected in any hydrolysate indicating cellobiase loading was appropriate. On the other hand, the 70°C treatment produced sugar yields similar to the corresponding 85°C treatment, and the 100°C treatment was only efficient on glucose production; it was clearly inferior in hemicellulose hydrolysis, which is in agreement with what was found in dwarf elephant grass *(2)*. In other words, high temperatures are helpful for cellulose hydrolysis but not for hemicellulose hydrolysis. The 1-min

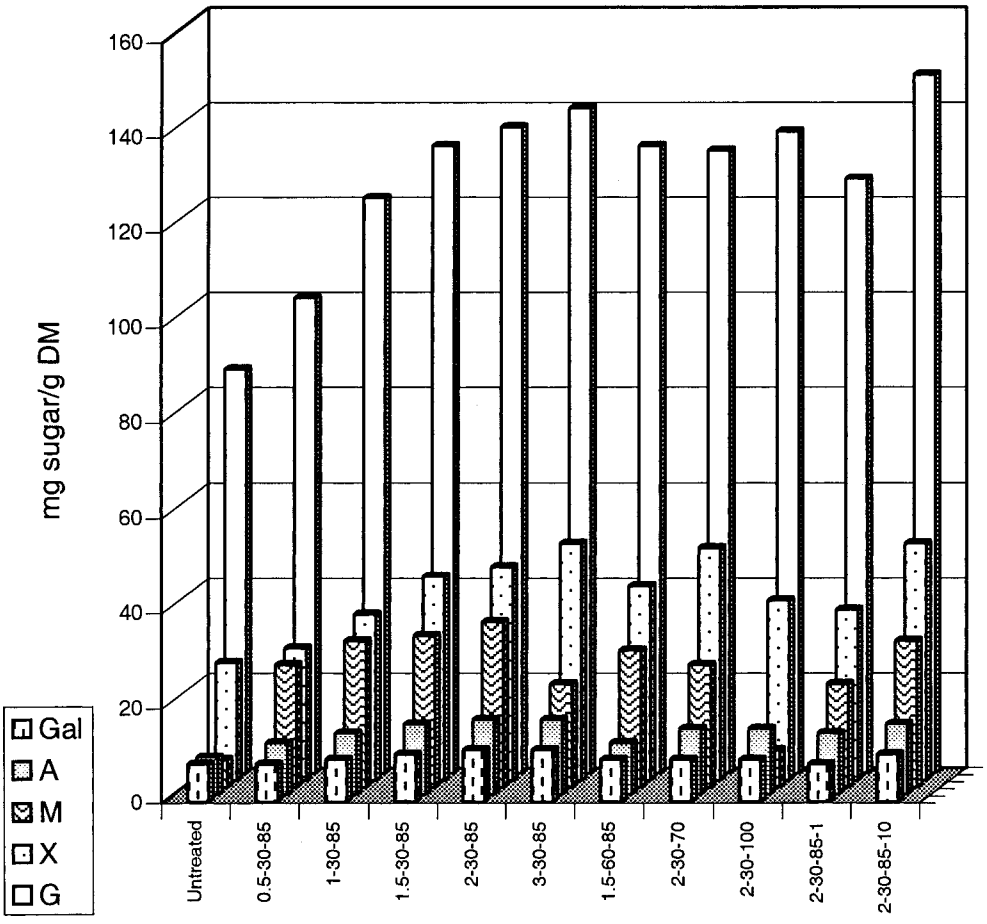

Fig. 4. Glucose (G), xylose (X), mannose (M), arabinose (A), and galactose (Gal) produced by enzymatic hydrolysis (24 h) of ALF. Enzyme mixture: Spezyme CP, Novozym 188 and Multifect XL.

treatment certainly produced lower yields than the corresponding 5-min treatment, and these were lower than the 10 min treatment. This effect can be better seen in the net sugars data presented in Fig. 5. These values correspond to the sugars produced by enzymatic hydrolysis. The 10-min treatment sugar yield was 7% greater than the corresponding 5 min treatment, and this was 11% greater than the corresponding 1-min treatment. However, what is important is the total sugar content of the hydrolysate. The data indicate that the 2–30–85, 3–30–85 and 2–30–85–10 treatments are equivalent (about 256 mg sugar/g DM). The advantage observed in the hydrolysis of the 10-min treatment sample is lost due to destruction of a major fraction of the initial glucose and fructose. Therefore, the 2–30–85 treatment (5 min) was chosen as the optimal condition due to lower ammonia loading and shorter time. An ammonia loading of 2 g/g dry matter looks as too much ammonia; however, it is possible to recover up to 99% of

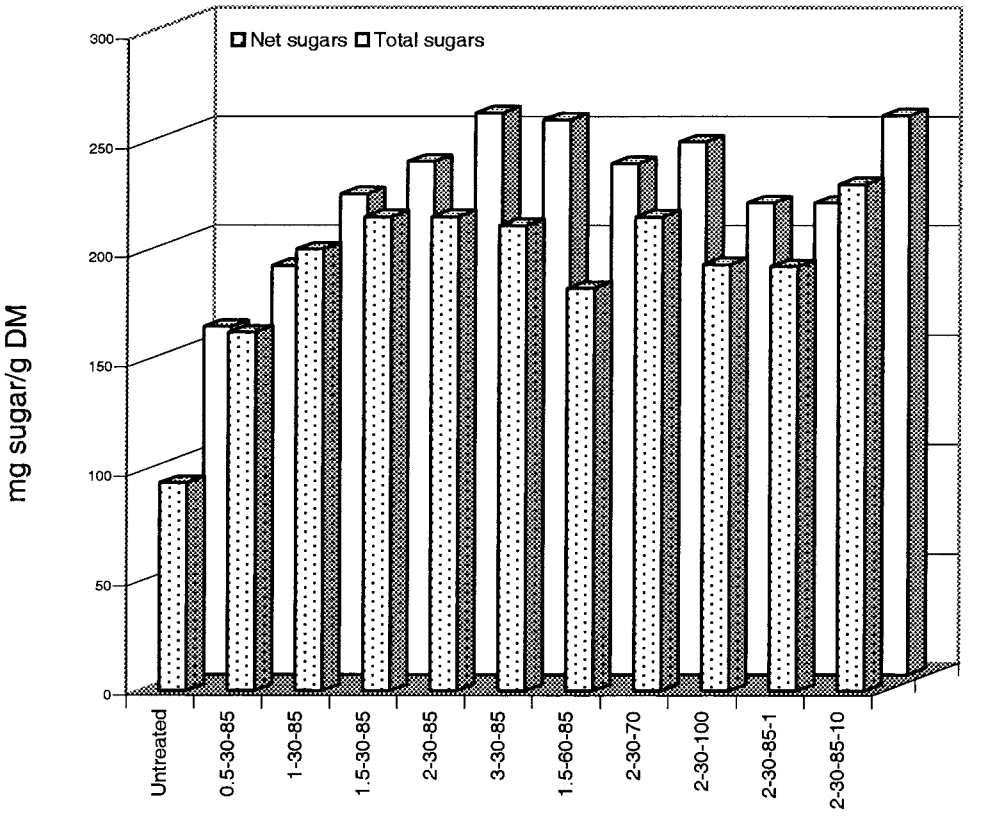

Fig. 5. Net and total sugars produced by enzymatic hydrolysis (24 h) of ALF. Enzyme mixture: Spezyme CP, Novozym 188 and Multifect XL. Total sugar includes initial sugar.

the ammonia. Process simulations (not yet published) based on the ASPEN software, which assume stripping the residual ammonia from the biomass followed by distillation of ammonia–water mixtures to recycle ammonia, are currently being performed to estimate the costs of ammonia recovery. Key simulation process variables include the scale of the process, moisture content of the incoming biomass feed, ammonia distillation column operating temperatures, and the desired amount of residual ammonia in the treated biomass. Given reasonable sets of values for these and other variables, ammonia recovery costs (capital and operating) are in the neighborhood of $10 per ton of biomass treated.

Conclusions

Glucose, fructose, and galactose concentrations decreased or disappeared with ammonia treatment. This was enhanced by increased temperature. Sucrose appeared to be formed from its monomers. Xylose concentration did not change with treatment, suggesting relatively high

stability compared with glucose, fructose, and galactose. Hemicellulose was solubilized but not hydrolyzed to monomers during the ammonia treatment. Ammonia treatments greatly increased susceptibility of alfalfa to fiber hydrolysis. The increase in sugar yield of treated alfalfa compared with untreated was 2.9 fold. The ammonia treatment increased the extent of hydrolysis of both cellulose and hemicellulose. Conversion of hemicellulose was relatively more enhanced than cellulose conversion with treatment.

Ammonia loading appeared as the main factor affecting sugar yields. A 76% of theoretical fiber conversion to sugars attained with cellulase and hemicellulase loadings of 5 FPU/g DM and 24 h hydrolysis time suggest great potential to produce sugars from ammonia-treated alfalfa for either animal feeding or fermentation uses. The optimal processing condition was 2 g ammonia/g DM–30% moisture–85°C–5 min.

Acknowledgments

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