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Enzymatic Hydrolysis of Ammonia-Treated Sugar Beet Pulp

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Abstract

Sugar beet pulp is a carbohydrate-rich coproduct generated by the table sugar industry. Beet pulp has shown promise as a feedstock for ethanol production using enzymes to hydrolyze polymeric carbohydrates and engineered bacteria to ferment sugars to ethanol. In this study, sugar beet pulp underwent an ammonia pressurization depressurization (APD) pretreatment in which the pulp was exploded by the sudden evaporation of ammonia in a reactor vessel. APD was found to substantially increase hydrolysis efficiency of the cellulose component, but when hemicellulose- and pectin-degrading enzymes were added, treated pulp hydrolysis was no better than the untreated control.

Index Entries: Biomass; sugar beet pulp; enzyme hydrolysis; ammonia pretreatment; fuel alcohol.

Introduction

Ethanol is used as an alternative fuel or an additive to currently used petroleum-based fuels and significantly reduces automobile emissions, decreases the dependence of the United States on foreign oil, and requires few alterations in current vehicle technology to be implemented. Currently, the United States has a production capacity of about 1.7 billion gal of ethanol/yr *(1)*. Much of this is used as a gasoline additive, in the form of 10% ethanol-blended fuels *(2)*. Almost all the ethanol produced today in the United States is from corn-based fermentations by yeast. This industry has been able to function with the help of residual coproducts and government subsidies, even though production costs are not currently competitive

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with petroleum *(3)*. Ethanol from corn is being used effectively as a fuel additive but this industry is limited in its growth potential because corn is an important human and animal food source, experiencing price fluctuations related to these food markets. Even if all the corn produced in the United States were devoted to ethanol production alone, only 15% of annual gasoline consumption could be replaced *(3)*. This fact underscores the importance of turning to alternative feedstocks for the production of ethanol. Many categories of feedstocks show potential for this application, including biomass energy crops, agricultural crop residues, woody biomass, and even municipal solid wastes *(3–5)*.

Sugar beet pulp is a coproduct of the table sugar (sucrose) industry and is the remaining plant fiber after the majority of the sucrose has been removed by processing. Sugar beets are farmed throughout the world in temperate climates; however, in the United States, sugar beet farming is concentrated in northern states west of the Mississippi, and also in Michigan and Ohio. Sucrose is also extracted from sugarcane, which is grown in tropical and subtropical areas of the world. Worldwide, about 35% of sucrose is derived from sugar beets, while the other 65% comes from sugarcane. However, in the United States approximately equal amounts of sucrose come from beet and cane sources, each contributing 3 to 4 million t of sugar annually *(6)*. In 1998, US sugar beet production topped 32 million t *(7)*. For a typical sugar beet processing plant, 250 kg of pressed beet pulp (75% [w/w] moisture) remain after the removal of sucrose from 1 t of sugar beets, equivalent to about 62.5 kg of dry matter beet pulp material *(8)*. Sugar beet pulp remains quite carbohydrate rich after processing (with about three-fourths of the dry matter weight being sugars), primarily owing to the sugars that compose the structural polymers, cellulose, hemicelluloses, and pectin *(8)*. Sugar beet pulp consists of 20–24% cellulose, 25–36% hemicellulose, 20–25% pectin or uronic acids, 1 to 2% lignin, and 7 to 8% protein, all expressed as a percentage of dry wt of total solids *(9,10)*. Sugar beet pulp has been used successfully for some time as a low-cost cattle feed, providing sucrose-processing plants a means for disposing of this residual material *(6)*. Taking into consideration that beet pulp is carbohydrate-rich, that sugar beet farming is a widespread and already mature industry, and that beet pulp is abundant and of relatively low value, this coproduct has potential for use as a renewable biomass feedstock for microbial fermentations to ethanol.

Successful conversion of beet pulp to ethanol has been accomplished by simultaneous saccharification and fermentation (SSF) using an engineered ethanologenic bacterium, *Escherichia coli* strain KO11 *(11)*. However, because this bacterium does not produce the cadre of enzymes necessary to degrade cellulose, hemicellulose, and pectin to their simple sugar units, exogenous enzymes are necessary. The level of commercially available fungal enzymes required to increase ethanol yields to distillable levels may be too high, because the enzymes are quite expensive. A variety of chemical pretreatments may be employed prior to SSF to render the

sugar beet pulp more easily hydrolyzed, thus reducing the amount of enzymes that must be added to achieve significant ethanol yields.

Cellulosic biomass materials typically require some sort of pretreatment to make the cellulose and other carbohydrate components reactive, or more susceptible, to enzymatic attack and use by microorganisms *(12–14)*. A successful pretreatment must economically promote the efficient conversion of carbohydrates to soluble sugars and should do so without formation of inhibitory products. The desired result of such a pretreatment is therefore to increase the effectiveness of the enzyme hydrolysis of biomass, producing more simple sugars than untreated biomass hydrolyzed under the same conditions. The ammonia pressurization depressurization (APD) treatment is a process for treating biomass with liquid ammonia at elevated pressures, followed by a rapid depressurization and violent evaporation of the ammonia. This process is a modification of the earlier ammonia fiber explosion (AFEX) process, which is well established as a successful biomass pretreatment. In contrast to AFEX, grinding of the materials is not employed and final reactor temperatures are well above freezing in APD *(15,16)*. AFEX has significantly increased the efficiency of hydrolysis of numerous forms of biomass, including wheat straw, rice straw, barley straw, corn stover, and alfalfa *(17)*; coastal bermudagrass and sugarcane bagasse *(18,19)*; switchgrass and rye straw *(20)*; and corn fiber *(21–23)*. APD works by two probable mechanisms. The rapidly evaporating ammonia rips apart the biomass fibers as it exits, exposing more surface area for enzymatic attack. Additionally, ammonia has the effect of decrystallizing the highly ordered cellulose strands found in lignocellulosic materials, allowing the cellulose to be more easily degraded during enzyme hydrolysis. Some delignification and hemicellulose hydrolysis effects of the APD treatment may also contribute to increased efficiency of hydrolysis *(17)*. The cost of ammonia pretreatment is estimated to be $20–$40 per dry t of biomass treated *(24)*.

In this study, we evaluate the effectiveness of APD in treating sugar beet pulp. This substrate has not previously been treated using this process, and in fact no such pectin-rich substrate has been APD treated to our knowledge. Therefore, two APD conditions were varied to establish the best APD operating conditions for sugar beet pulp. Hydrolyses with added commercially available fungal enzymes were done to determine the ease of hydrolysis of treated vs untreated pulp. Statistical analysis was performed to indicate the significance of the various treatment effects.

Materials and Methods

Sugar Beet Pulp

Beet pulp from the common sugar beet *Beta vulgaris* was provided by Monitor Sugar (Bay City, MI) in a pressed form containing approx 25% (w/v) solids. Beet pulp was stored at –20°C when not in use. Prior to APD processing, beet pulp was dried by baking at 45°C for 24 h and was then rehydrated

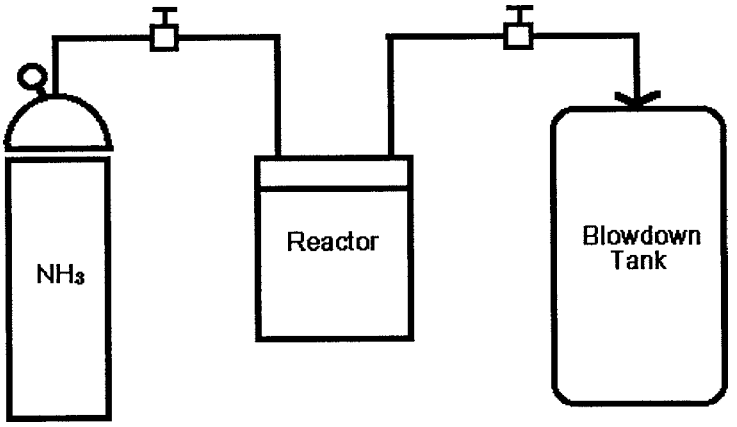

Fig. 1. Diagram of the APD reactor apparatus.

to the desired moisture content levels for APD treatment. Beet pulp fibers were used at the original size at which they emerged from processing, with no further grinding or milling. Fibers were in the size range of several millimeters in width and length.

Ammonia Pressurization Depressurization

The APD reactor apparatus (Fig. 1) was a modified steel pressure vessel attached to an ammonia source and a blowdown tank. In this study, two APD treatment conditions were varied: the beet pulp moisture content and the added ammonia load. Sugar beet pulp was treated at moisture contents of 50, 66, and 75% (w/w), and the ratio of ammonia load to beet pulp was set at levels of 0.5:1, 0.75:1, and 1:1 for each moisture level. Other APD treatment conditions were held constant, with a temperature of 80°C and a treatment time of 5 min for all samples. Following APD treatment, residual ammonia in samples was allowed to evaporate for 24 h in a fume hood, and the treated material was then stored at –20°C until used.

Enzyme Hydrolyses

Beet pulp underwent enzymatic hydrolysis at 40°C with a 5% (w/w) solids load, in a 0.05 *M* citrate buffer solution (pH 4.8). Hydrolysis proceeded for 48 h, and periodic samples taken for analysis were placed in a boiling water bath for 15 min to deactivate enzymes, which were then stored at –20°C. Sodium azide (0.15% [w/v]) was added to prevent microbial contamination. Three commercially available enzyme mixtures provided by Novo Nordisk (Franklinton, NC) were used for pulp saccharification. Celluclast 1.5L FG contains about 1500 Novo Cellulase U/mL (Novo Nordisk assay), or 102 filter paper units (FPU) of activity/mL *(8)*. Novozym 431 contains approx 250 cellobiase units (CBU)/mL (Novo Nordisk assay). Viscozyme L contains about 32 hemicellulase units (HU) and 2300 poly-

galacturonase units (PGU)/mL *(8)*. Two sets of hydrolysis experiments were performed; the first set used only cellulase and cellobiase, and the second set added the hemicellulase/polygalacturonase. Enzyme loadings were kept constant at 4.2 FPU, 28.4 CBU, 0.85 HU, and 60.2 PGU/g of dry wt of sugar beet pulp.

Analytical Methods

Scanning electron microscopy (SEM) was used to examine the microscopic appearance of treated and untreated sugar beet pulp fibers. Beet fibers were fixed in 5% glutaraldehyde, dehydrated in a graded ethanol series (35, 50, 85, 95, 100, 100, and 100%), critical point dried, and sputter coated with 30 nm of gold. Multiple samples were observed over a range of magnifications on an Amray AMR 1200 microscope.

A modification of the dinitrosalicylic acid (DNS) assay *(25)* was used to measure the amount of soluble reducing sugars released during hydrolysis. Samples were filtered through a 0.2-µm syringe filter prior to reaction with DNS reagent (containing 1.0 g/L of 3,5-DNS and 300 g/L of sodium potassium tartrate in a 0.4 *M* NaOH solution). Sugars were reported in reducing sugar units using glucose, arabinose, and galacturonic acid to create the standard calibration curve. A factorial design analysis of variance (ANOVA) test was performed on the glucose equivalent yields (only from hydrolyses with hemicellulase/pectinase) to determine statistical differences from treatment effects, and a Tukey test for multiple comparisons was done to ascertain where differences lay. The Minitab statistical package (version 11) was used for all statistical applications.

High-performance liquid chromatography (HPLC) was performed on selected samples to measure specific sugar contents and organic acids. Samples analyzed for sugars by HPLC were eluted for 60 min with a Milli-Q water mobile phase through a Bio-Rad HPX 87-P column heated at 85°C, and sugars were detected by a refractive index detector. For detection of organic acids, a Bio-Rad HPX-87H column was heated at 65°C, with a mobile phase of 10 m*M* H_2SO_4 at 0.6 mL/min. Quantification was achieved by chromatogram peak area measurement with external standards.

Samples were submitted to an independent testing lab (Agri-King, Fulton, IL) for compositional analysis according to Official Methods of Analysis of AOAC International.

Results and Discussion

Effect of APD on Sugar Beet Pulp Structure and Composition

The APD treatment had an obvious physical effect on sugar beet pulp fibers. The size of the particles was somewhat reduced and the color of treated beet fibers was substantially darkened. This dark color persisted through hydrolysis experiments, giving the hydrolysis slurry a much darker tint than untreated controls.

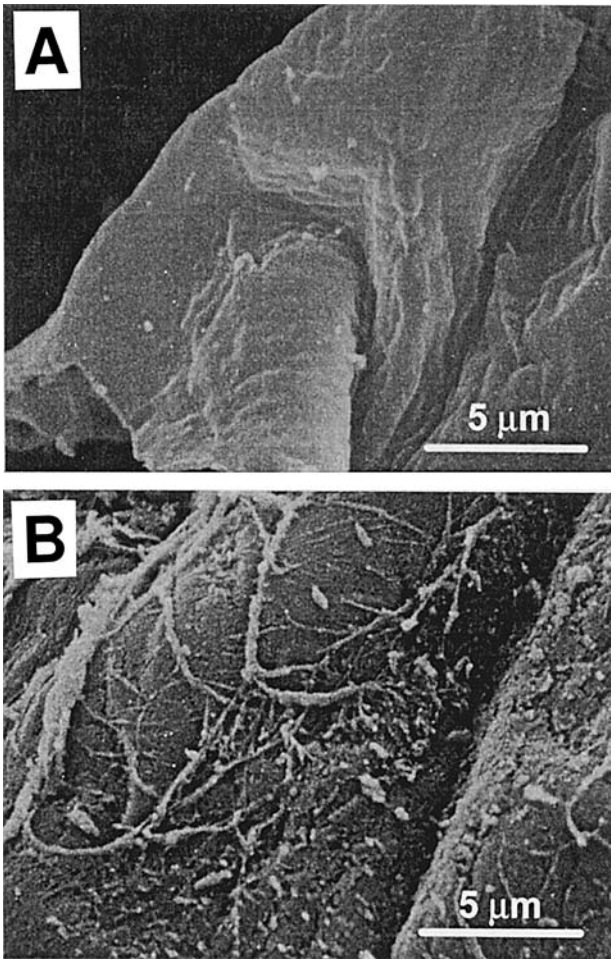

Fig. 2. Scanning electron micrographs of **(A)** untreated and **(B)** APD-treated sugar beet pulp.

Using SEM, the microscopic surfaces of treated and untreated pulp fibers were compared. Examination at an original magnification of ×5000 or higher showed great differences between treated and untreated fibers. As seen in Fig. 2, untreated pulp fibers had a smooth and intact surface, very little debris, and well-defined edges and folds. By contrast, APD-treated pulp fibers exhibited a disrupted surface with large amounts of stringy and fibrous debris, as well as small tears and holes in the pulp surface. The use of SEM with other ammonia-treated biomass types has previously shown a similar disruption of fiber but typically on a larger scale, with splitting fibers parallel to the longitudinal axis *(17)*.

Analysis of samples after treatment indicated that there were no major changes in the cellulose component or in the levels of many minerals. The crude protein content increased three- to fourfold based on the Kjeldahl

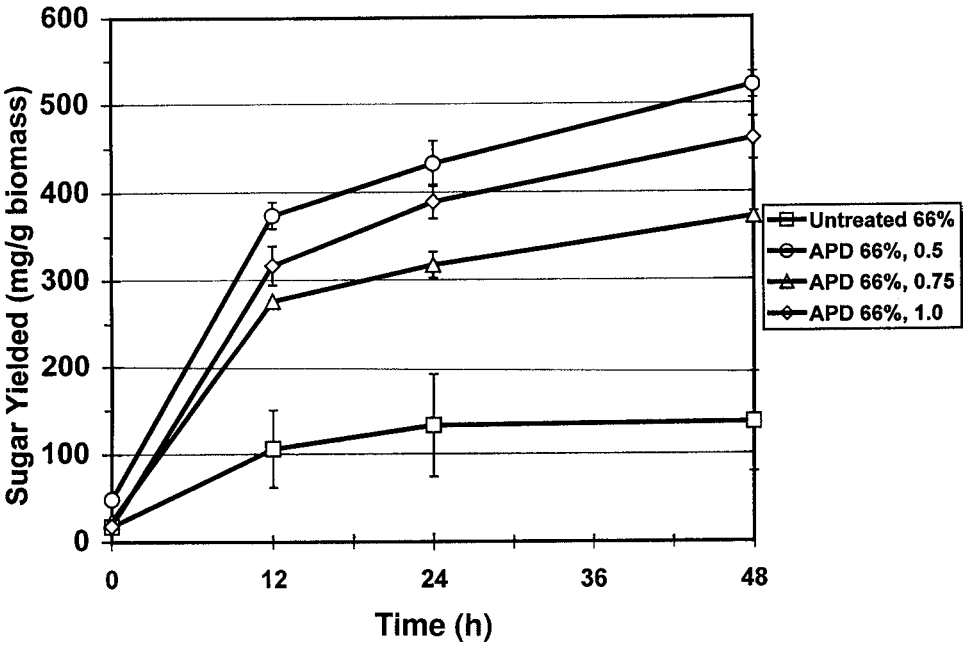

Fig. 3. Reducing sugars yielded from the enzymatic hydrolysis of APD-treated and untreated sugar beet pulp (66% moisture condition) using cellulase and celllobiase.

method, a large increase that has been observed in the previous ammonia treatment of other types of biomass *(15,19)*. At the lowest ammonia load (0.5:1), hemicellulose and pectin contents were approximately equal to those of the untreated controls. However, as ammonia levels increased to 0.75:1 and 1:1, a concomitant decrease in both hemicellulose and pectin was observed. At these higher ammonia loads, 50% of the hemicellulose and 20% of the pectin was destroyed or removed during processing. Under all APD conditions examined, lignin concentrations were found to be three-fold higher than for the untreated controls.

Hydrolyses with Cellulase

The first objective was to evaluate the effectiveness of enzymatic hydrolysis of APD-treated and untreated sugar beet pulp. In previous studies, APD has been evaluated by hydrolyses employing cellulase and cellobiase enzymes to determine the effectiveness of cellulose degradation *(16,18,20,26–28)*. Therefore, hydrolysis experiments on treated and untreated SBP were performed with additions of Celluclast 1.5L FG and Novozym 431. Figure 3 shows the reducing sugars yielded from hydrolysis of APD-treated pulp with a moisture content of 66% and varying ammonia loads. As expected, APD substantially increased hydrolysis yields and rates over those of untreated controls. APD-treated pulp produced reducing sugar yields two- to threefold higher than untreated pulp. Previous studies

of ammonia-treated agricultural residues reported four- to fivefold sugar yields increases over untreated materials *(17,20)*. Volumetric sugar yields from the first 12 h of hydrolysis were also higher in APD-treated sugar beet pulp, showing a 2.5- to 3.5-fold increase over untreated material. The lowest ammonia loading resulted in the greatest release of reducing sugars. APD-treated material reached about 85% of its total sugar yield after 24 h of enzyme hydrolysis in these experiments. Similar results were observed in cellulase hydrolyses of APD-treated and untreated pulp with moisture contents of 50 and 75% (not shown). Note that all APD-treated hydrolyses resulted in sugar yields higher than the maximum theoretical yield, when only cellulose was hydrolyzed. Complete hydrolysis of the cellulose component would yield approx 240 mg of reducing sugar/g of biomass. It is clear from these hydrolyses, coupled with the SEM analysis, that APD treatment disrupts the fibrous structure and facilitates enzymatic hydrolysis with cellulases. Additional hemicellulose or pectin degradation must also occur to obtain higher yields than expected from cellulose hydrolysis alone.

Hydrolyses with Cellulase and Added Hemicellulase/Pectinase

Susceptibility to enzyme hydrolysis was increased in cellulase hydrolyses of ammonia-treated beet pulp; however, cellulose makes up only about one-third of the total carbohydrates in sugar beet pulp. The cellulase hydrolyses neglect to consider the hydrolysis of the pectin and hemicellulose components, which make up roughly the other two-thirds of the carbohydrates in SBP. For the overall process efficiency of sugar beet pulp hydrolysis to be acceptable, all three large carbohydrate components must be considered. Therefore, further hydrolysis experiments were carried out with the addition of Viscozyme L, an enzyme mixture possessing hemicellulase, pectinase, and polygalacturonase activities. All sets of treated and untreated samples were hydrolyzed under identical conditions with fungal enzymes Celluclast 1.5L FG, Novozym 431, and Viscozyme L.

The results of these sets of hydrolyses were quite different from the previous cellulase- and cellobiase-only experiments, indicating that treated beet pulp consistently produced lower reducing sugar yields than untreated controls, regardless of APD parameters used. Table 1 summarizes the average reducing sugar yields from treated and untreated sugar beet pulp. Untreated beet pulp outproduced treated pulp in reducing sugar yields by about 100 mg/g of biomass, or greater. Among APD-treated samples, those with the lower ammonia load of 0.5:1 were associated with higher sugar yields on average, although differences were relatively small (50 mg/g of biomass) (Fig. 4). APD-treated pulp at 75% moisture produced lower average sugar yields as ammonia loads increased, suggesting an association between higher ammonia loads and poor hydrolysis.

A factorial design ANOVA (two-way ANOVA with replication) was performed on hydrolysis DNS data to determine whether differences

Table 1
Average Reducing Sugars Yielded
from APD-Treated and Untreated Sugar Beet Pulp During Hydrolysis
with Cellulase, Cellobiase, and Pectinase (± Standard Error Term)

Factor 1: SBP moisture	Factor 2: Ammonia load			
	Untreated	0.5:1	0.75:1	1:1
50%	786 ± 35 mg/g	608 ± 32	546 ± 37	548 ± 37
66%	700 ± 85	605 ± 52	554 ± 40	565 ± 19
75%	743 ± 6	561 ± 37	505 ± 26	350 ± 127

between APD-treated and untreated pulp sugar yields were significant, and what effect moisture and ammonia load had on effectiveness of hydrolysis. Table 2 summarizes the statistical results. Moisture content was not found to be a significant factor, with a p value of 0.131. Therefore, the three moisture levels, 50, 66, and 75%, produced statistically equivalent results and are, for all practical purposes, the same. Ammonia load was found to be a significant factor and had a p-value of <0.0005. No interaction was found to exist between the moisture content and ammonia load, because the interaction term had a high p value of 0.434. A Tukey test for multiple comparisons was performed to determine where the differences lay among the levels of ammonia load. The Tukey test revealed that the untreated sugar beet pulp yielded significantly higher reducing sugar yields than any of the treated pulp material and also that no difference existed among the APD-treated pulp samples owing to ammonia load. In other words, the three levels of ammonia load were found to have essentially equivalent effects on hydrolysis effectiveness.

When compared with the untreated control, the decrease in total reducing sugars at the two highest ammonia loads can be explained by the decrease in hemicellulose and pectin content. There is simply less carbohydrate available for enzymatic hydrolysis. However, the decrease in total reducing sugars at the lowest ammonia loading cannot be explained by a difference in the starting carbohydrate content. Cellulose, hemicellulose, and pectin content were essentially identical between the 0.5:1 ammonia load and the untreated controls. To elucidate the profile of carbohydrates released during enzymatic hydrolysis, HPLC analysis was performed.

HPLC analysis provided detection and quantification of specific sugars (cellobiose, glucose, arabinose, and galacturonic acid) throughout enzymatic hydrolysis, making it possible to measure differences in the release of simple sugars from APD-treated and untreated sugar beet pulp. During the first 12 h of enzymatic hydrolysis, the carbohydrate profiles of untreated and 0.5:1 ammonia treated pulp were very similar (Fig. 5). After 12 h more galacturonic acid and arabinose were released from the untreated samples. Approximately 66 mg/g more galacturonic acid and 55 mg/g

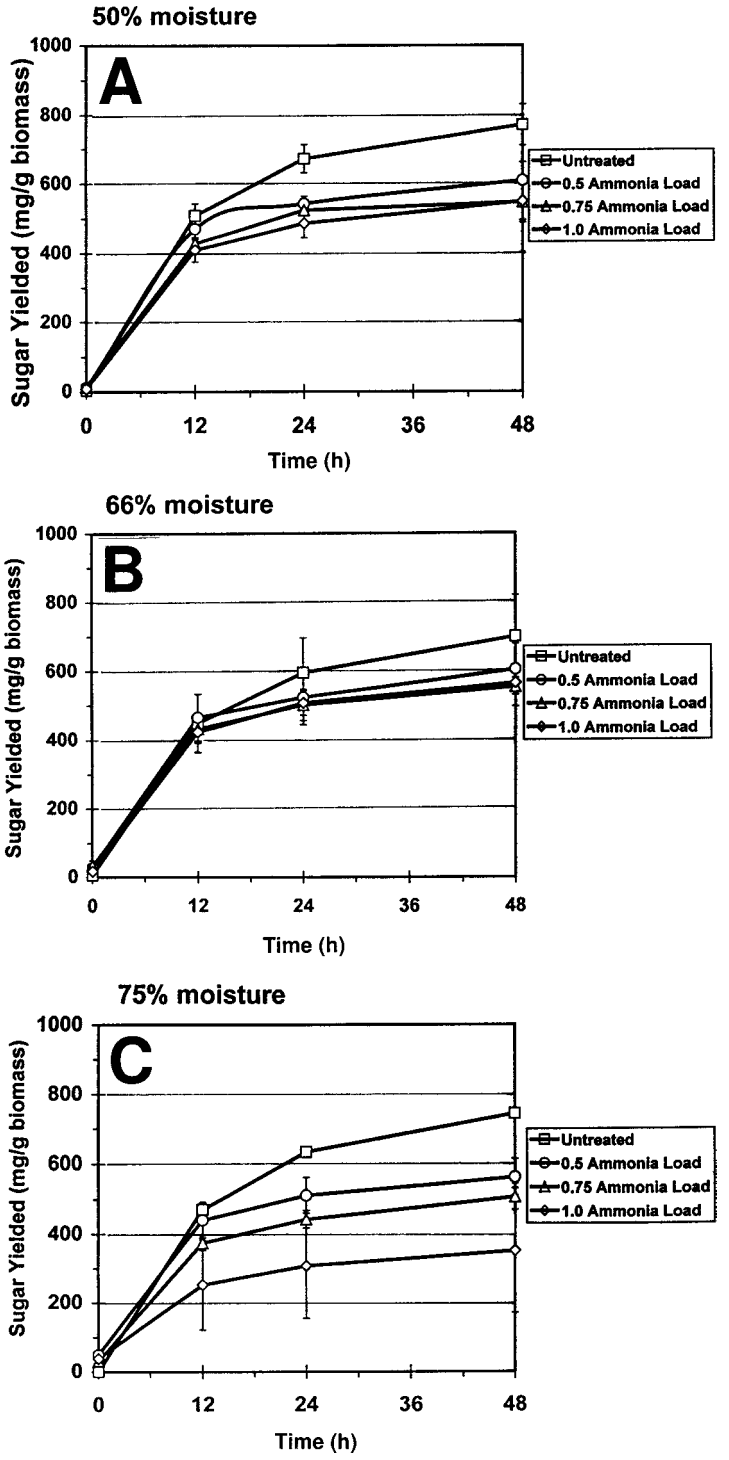

Fig. 4. Reducing sugars yielded from the enzymatic hydrolysis of APD-treated and untreated sugar beet pulp at **(A)** 50%, **(B)** 66%, and **(C)** 75% moisture contents, using cellulase, cellobiase, and pectinase enzymes.

Table 2
ANOVA Table Summarizing Factorial ANOVA Results for the Factors of Moisture Content and Ammonia Load, and Their Interaction for APD Treatment of Sugar Beet Pulp

Source	DF	Adjusted MS	*F*-Ratio	*p*-Value
Moisture content	2	20,142	2.22	0.131
Ammonia load	3	100,440	11.06	0.000
Moisture × ammonia	6	9291	1.02	0.434
Error	24	9082		

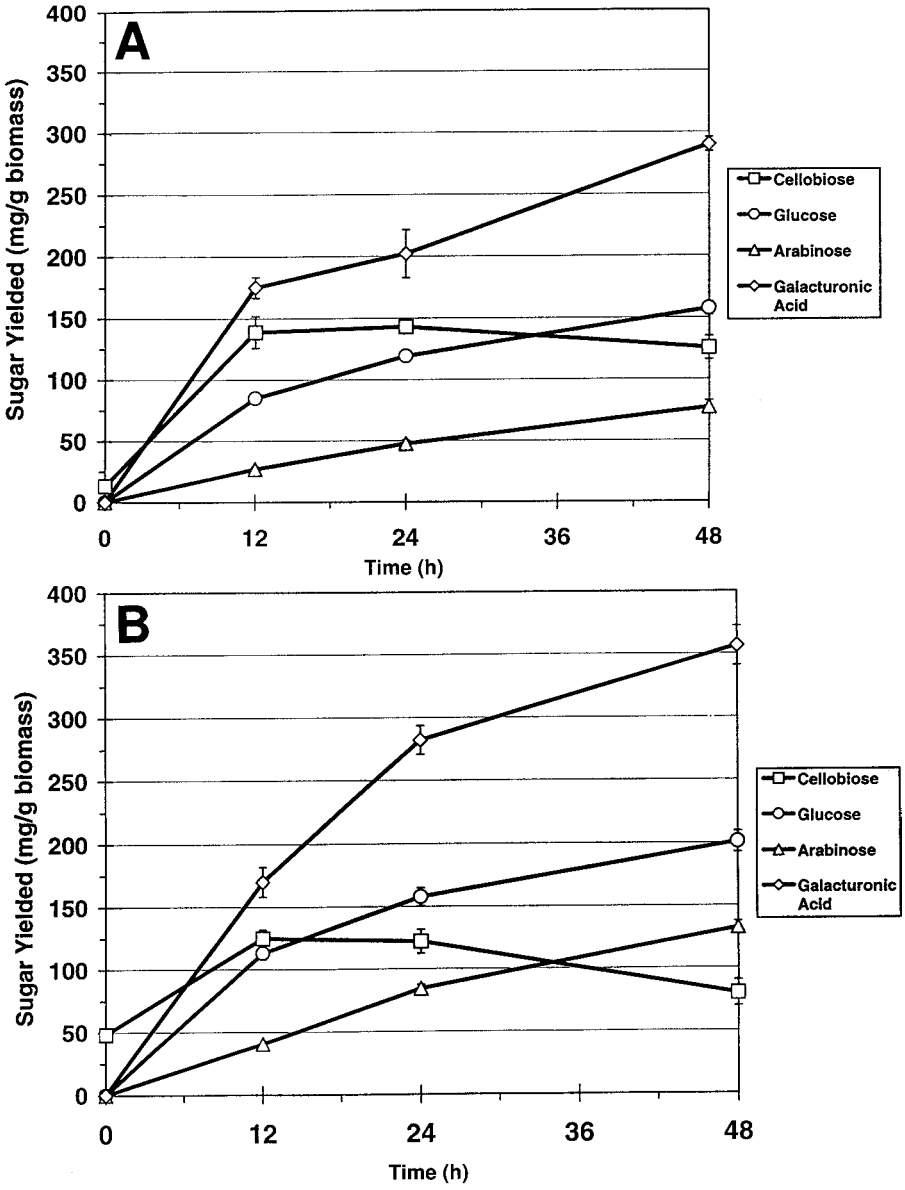

Fig. 5. Sugars yielded from the enzymatic hydrolysis of **(A)** APD-treated and **(B)** untreated sugar beet pulp using cellulase, cellobiase, and pectinase, as determined by HPLC (± standard error term).

more arabinose were released by 48 h. The total amount of carbohydrates quantified by HPLC was 650 mg/g for the treated pulp and 770 mg/g for the untreated pulp, ranges within those obtained by the DNS analysis as well (Fig. 4).

Conclusion

In summary, the APD processing of sugar beet pulp appears to have disrupted the structure as evidenced by SEM analysis and significantly enhanced the enzymatic hydrolysis when cellulases and cellobiase were used. Total cellulose content remained unchanged during APD treatment and lignin content increased threefold for all ammonia levels examined. When a third enzyme mixture of hemicellulases and pectinases was added, carbohydrate yields for untreated pulp were higher than for any of the ammonia treatments. Because hemicellulose and pectin contents of the lowest ammonia loading and untreated pulp were essentially identical, APD appears to have altered the structure of pectin and/or hemicellulose, thereby decreasing effective hydrolysis by the enzymes used in this study. Perhaps at even lower ammonia loads, the physical structure of the pulp could be altered without reducing degradation by pectinase enzymes. At ammonia loads of 0.75:1 or higher, hemicellulose or pectin appear to be degraded or destroyed.

Studies on composition of sugar beet-cell walls indicate that the separation of lignocellulosic components is achieved by solubilization of pectin, which in turn is a result of arabinan hydrolysis *(29,30)*. Because galacturonic acid exclusively originates from pectin, an increase in galacturonic acid indicates better hydrolysis of the pectin component. Higher concentrations of both galacturonic acid and arabinose were found in enzymatic hydrolysates from untreated pulp, indicating more effective hydrolysis of the pectin component. Sugar beet pectin is a heteropolysaccharide composed of a "smooth" backbone of polygalacturonan and resistant "hairy" regions consisting of arabinan and arabinogalactan. These "hairy" regions are bound to the backbone by galactopyranosil-rhamnopyranosil or arabinofuranosil-rhamnopyranosil linkages *(30)*. Pectin solubilization is obtained when the glycosidic linkages are broken in the arabinan hairy region. In sugar beets, ferulic acid is esterified to the arabinan and (galacto) arabinan side chains, making it possible to crosslink pectins *(31)*. Such crosslinking of arabinan and galactan side chains by ferulate dehydrodimers is responsible for decreased digestibility by ruminants *(32)*. Further studies are needed to determine whether the degree of crosslinking of side chains is increased in APD-treated pulp, thus providing one explanation for the decrease in enzymatic hydrolysis of APD-treated beet pulp when compared with untreated pulp. However, under the conditions examined, APD treatment only enhances degradation of cellulose and offers no advantage for enzymatic hydrolysis of the pectin and hemicellulose fractions.

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