ENZYMATIC HYDROLYSIS OF SUGARBEET PULP FOR CELLULOSE ISOLATION AND ETHANOL PRODUCTION

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Introduction

With volatile fuel and energy prices and renewable fuel mandates such as those in the 2007 Energy Independence and Security Act, there is a greater interest in finding new feedstocks for biofuel production. Sugarbeet pulp has not received a great deal of attention because of its limited supply on a national basis. On a regional basis, however, Minnesota and North Dakota combined typically produce more than 50% of the total US sugarbeets [1]. Ethanol from non-sucrose sugars in beet pulp could produce regionally up to 75-90 million gallons of ethanol per year. This could potentially be combined with sucrose ethanol from excess beet production if fermentation facilities were developed.

As a potential feedstock, sugarbeet pulp has some significant logistical advantages over more conventional biomass sources such as corn stover, wheat straw, or perennial bioenergy crops. Biomass harvest, storage and transportation may all be limiting factors in conventional biomass processing while beet pulp requires little or no extra cost or technical development in these areas. In addition, the relatively unique composition of beet pulp gives it some processing advantages. Other biomass feedstocks require expensive thermochemical pretreatment prior to enzymatic hydrolysis of the cellulose fraction [2]. Sugarbeet pulp has a large percentage of pectin and hemicellulose that may be hydrolyzed using commercial enzyme mixtures without the need for thermochemical processing [3, 4].

Potential challenges of utilizing beet pulp for ethanol production include a unique and complex sugar stream [3]. Sugars hydrolyzed from hemicellulose and pectin include galactose, arabinose, and galacturonic acid, among others. Such sugars may be fermented to ethanol but not by conventional yeasts used for other ethanol fermentations [5-7]. The organisms that can ferment these sugars generally do so with lower yields and tolerate lower final ethanol concentrations (<5%) [8]. Hydrolysis solid loading requirements and these lower ethanol tolerances lead to larger reactor volumes and higher equipment and distillation costs.

Cellulose hydrolysis yields glucose, a sugar readily fermented by conventional yeasts to concentrations greater than 13%. One possible way of circumventing the difficulty in achieving higher ethanol titers from beet pulp hydrolyzates would be through separate hydrolysis and fermentation of the various carbohydrate components. Pectin and hemicellulose could be enzymatically hydrolyzed leaving a cellulose-enriched component [3, 4]. The galactose, arabinose, and pectin-derived galacturonic acid could be subsequently fermented with the recombinant *E. coli* K011 while the cellulose fraction could be processed with a conventional *Saccharomyces cerevisiae* strain in a higher-solids simultaneous saccharification and fermentation (SSF) process to reach higher ethanol titers. The primary objective of this research was to explore the technical feasibility of using sequential enzyme treatments for separation of sugar streams derived from cellulose from sugars derived from hemicellulose and pectin.

Materials and Methods

Sugarbeet Pulp. Wet and pressed sugar beet pulp were obtained from American Crystal Sugar Company in Moorhead, MN. Moisture contents were found to be 88% and 71% (wet basis), respectively. Samples were stored at -20°C.

Enzymes. All enzymes used were produced by Novozymes Corporation (Bagsvaerd, Denmark) and purchased from Sigma-Aldrich (St. Louis, MO). Two commercial preparations of enzymes (Viscozyme L and Pectinex Ultra) were used to hydrolyze pectin and hemicellulose within the sugarbeet pulp. Both products are composed of a combination of enzymes with hemicellulase, pectinase, and cellulase activities. The enzymes were tested both independently and in combination to quantify interaction. Flavourzyme is a fungal protease that was used to hydrolyze the protein component of beet pulp.

Hydrolysis. Sugar beet pulp was sterilized by autoclaving (121°C, 20 min) prior to enzyme treatments. For hemicellulase/pectinase treatments, beet pulp (1.235 dry g) was mixed with 50 mM citrate buffer (pH 5.0) in 125-mL Erlenmeyer flasks at a loading rate of 5% weight per volume. Hydrolysis was carried out in a water bath at 40°C and 100 rpm. Samples (2 ml) were taken 1-2 times per day for up to 48 hours. All treatments were conducted

in triplicate. Protease (Flavourzyme) treatments were carried out in a similar manner using 100 mM phosphate buffer (pH 6) with temperature and shaking rates of 50°C and 100 rpm, respectively.

Pectinex and Viscozyme were tested both individually and in combinations with a combined enzyme loading rate of 100 µL/dry g. Pectinex was also tested at loading rates up to 250 µL/dry g. Individual sugar yields were compared to determine which enzyme was most effective at separating the cellulose from the other pulp components.

Flavourzyme was used at a loading rate of 8.1 µL/dry g. Samples were taken for HPLC analysis after 24 hrs and the remaining solids were vacuum filtered using a Buchner Funnel and Whatman No. 41 filter paper. Filtration was done in a sterile hood and remaining solids were returned to their original flasks for further hydrolysis of pectin and hemicellulose. Residual solids were added to citric acid buffer (50 mM, pH 5; 25 ml/original dry g) and heated at 90°C for 20 min to deactivate any remaining protease. Pectinex was then added at a loading rate of 100 µL/original dry g. Flasks were then incubated in a water bath at 40°C and 100 rpm; samples were taken for HPLC analysis at 0, 6, 19, and 24 hrs.

Sugar Analysis. HPLC was used to quantify sugar yield following enzymatic hydrolysis. Samples were centrifuged and filtered through a 0.2-µm nylon filter (Pall Corporation, West Chester, PA) prior to HPLC analysis. Cellobiose, glucose, arabinose, galactose, and fructose were quantified using a Waters (Milford, MA) HPLC and refractive index detector. Sugars were separated using a Bio-Rad (Hercules, CA) Aminex HPX-87P column with a mobile phase of water at a flow of 0.6 mL/min; the column and detector temperatures were 50°C and 85°C, respectively. Cellulosic glucose concentrations were calculated by subtracting sucrose contribution to soluble glucose; sucrose hydrolyzes to equimolar quantities of fructose and glucose so sucrose contribution was estimated by fructose concentration. Galacturonic acid was quantified and separated using a Waters HPLC and photodiode array detector (210 nm wavelength). Separation was done using a Bio-Rad Aminex 87H column with a mobile phase of 5 mM sulfuric acid at a constant flow of 0.6 mL/min at 60°C. All sugars were quantified using a 3-point external standard curve for each component. Saccharification yields were calculated as a percent of theoretical based on published composition data [4].

Results and Discussion

Figure 1 shows sugar yields from hydrolysis with Viscozyme, Pectinex, and combinations of the two products. Yields of galacturonic acid were significantly higher than 100% of theoretical; it is assumed that this is the result of compositional differences between local beet pulp and published data. Pectin and hemicellulose were hydrolyzed to a greater extent with Viscozyme (4:0) than with Pectinex (0:4) as shown by galacturonic acid and arabinose/galactose yields, respectively (Figure 1). Viscozyme also produced greater free glucose yields from the cellulose component than did Pectinex but these differences were mostly due to higher concentrations of cellobiose in Pectinex-treated samples. Combinations of the two enzymes yielded no significant difference in hydrolysis indicating that both products hydrolyze similar subcomponents of the hemicellulose and pectin.

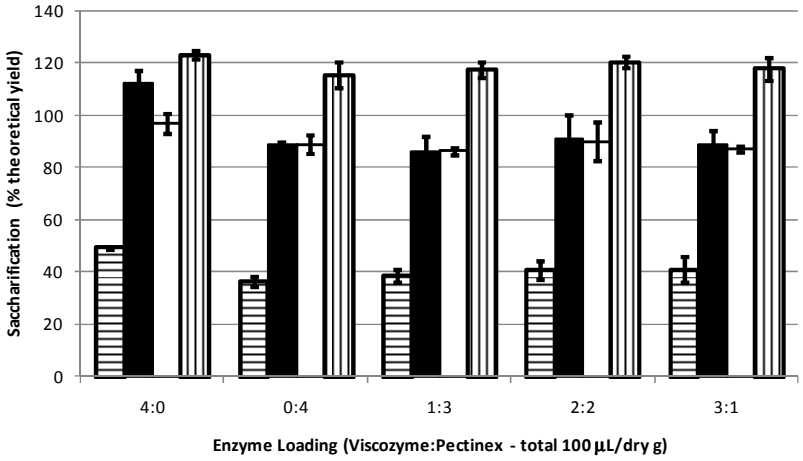

Figure 1. Saccharification (after 48 hr) as percentage of theoretical yields using combinations of Viscozyme and Pectinex. ▤ Glucose, ■ Galactose, □ Arabinose, ▥ Galacturonic Acid.

Based on this data, Viscozyme hydrolyzed pectin and hemicellulose better than Pectinex. Project goals also included separation of the cellulose component from the hemicellulose and pectin portions. Viscozyme was tested against increasing loading rates of Pectinex to achieve similar yields of hemicellulose and pectin sugars. Table 1 shows results of these experiments. Free glucose concentrations were significantly lower using Pectinex, but cellobiose concentrations were significantly higher at all loadings tested. Cellulose hydrolyzes to the glucose dimer cellobiose and then into glucose. To determine how much cellulose had been hydrolyzed, free glucose concentrations were added to the glucose content of the measured cellobiose concentrations (1.053 g glucose/g cellobiose). Table 1 shows that increasing Pectinex loading rates to 250 µL/dry g did increase yields of other sugars to levels comparable to those achieved with Viscozyme at 100µL/dry g. At this level, however, cellulose hydrolysis was also greater as shown by the higher total glucose concentrations. These results indicate that given similar hemicellulose and pectin hydrolysis, Viscozyme hydrolyzes less cellulose allowing to be separated for downstream hydrolysis and yeast fermentation.

Table 1. Glucose, cellobiose, and other sugar yields Viscozyme and Pectinex treatments.

Enzyme	Loading Rate (µL/dry g)	Free Glucose (g/L)	Cellobiose-Glucose (g/L)	Total Glucose (g/L)	Other Sugars (g/L)	Glucose : Other Sugars
Viscozyme	100	2.410	0.282	2.692	13.936	0.193
Pectinex	100	0.042	1.985	2.027	12.161	0.167
Pectinex	150	0.355	1.776	2.131	12.589	0.169
Pectinex	200	1.007	1.740	2.747	13.457	0.204
Pectinex	250	1.304	1.663	2.966	13.745	0.216

The protease Flavourzyme was used prior to Pectinex treatment to determine if protein hydrolysis would improve hydrolysis and separation of the remaining pulp components. Figure 2 shows individual sugar yields for Pectinex treatments and Pectinex treatments preceded by Flavourzyme treatment. Yields for all sugars were significantly lower following Flavourzyme treatment. Destruction of hemicellulases and pectinases by residual proteases should have been minimized or eliminated through filtration and inactivation by 90°C heat treatment of solids prior to hemicellulase/pectinase addition. In addition, Flavourzyme and Pectinex have different optimum temperatures and pHs. Further explanation of the cause for inhibition of further hydrolysis was not explored. Flavourzyme pretreatment failed to achieve the goal of increasing hemicellulose and pectin hydrolysis and will not be used in the future.

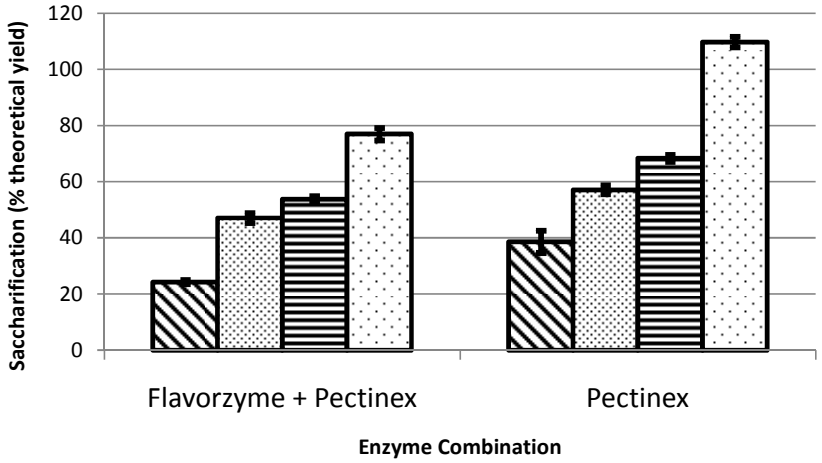

Figure 2. Saccharification (after 48 hr)as a percentages of theoretical yields using Pectinex with or without a Flavourzyme pretreatment of sugar beet pulp. ▨ Glucose, ▩ Galactose, ▤ Arabinose, ▢ Galacturonic Acid

Initial experiments were completed using wet beet pulp. Use of wet pulp would require less processing but would limit sucrose recovery; it was therefore deemed less desirable than pressed pulp as a feedstock. Pressed pulp and wet pulp were tested at 5% solids loadings with Viscozyme to confirm that earlier results were valid for pressed pulp. Results are shown in Figure 3. Use of Pressed Pulp resulted in increased galacturonic acid yields with no significant difference in yields for other sugars.

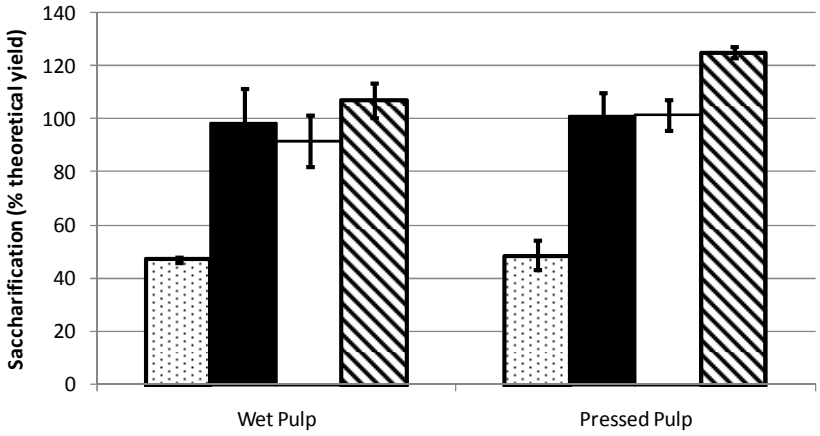

Figure 3: Sugar yields (48 hrs) for hydrolysis of wet and pressed sugar beet pulp at 5% solids loading rates. All samples were treated with Viscozyme at 100 µl/dry g.
Glucose, Galactose, Arabinose, Galacturonic Acid

With total sugar concentrations in the beet pulp hydrolyzate less than 17 g/L, increased solids loading rates were tested to determine the impact on hydrolysis. Figure 4 shows that increasing solids loading rates up to 8% had little impact on individual sugar yields.

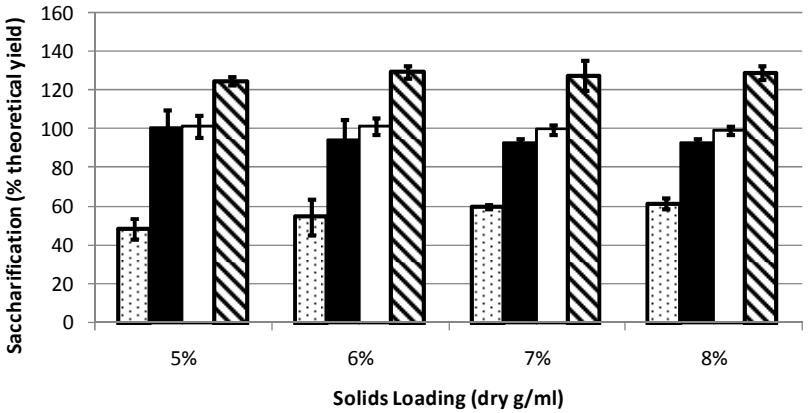

Figure 4: Sugar yields (48 hrs) for hydrolysis of pressed sugar beet pulp with increasing solids loading rates. All samples were treated with Viscozyme at 100 µl/dry g.
Glucose, Galactose, Arabinose, Galacturonic Acid

Conclusions

Enzymes with hemicellulase and pectinase enzymes effectively hydrolyzed beet pulp without thermochemical pretreatment. Protease treatment was not effective at increasing subsequent sugar yields from hemicellulase/pectinase treatment. Cellulase activity in the hemicellulase/pectinase enzyme products led to cellulose hydrolysis levels of approximately 40% of theoretical values. Given the relatively high level of cellulase activity in these products, it does not appear feasible to effectively separate the cellulose component by specifically solubilizing the pectin and hemicellulose. Other options should be explored to take advantage of the higher yields and ethanol tolerance of yeast fermentations.

Increasing solids loading rates to 8% had little impact on sugar yields but increased yielded total concentrations to greater than 50 g/L. Hydrolysis results with wet and pressed sugarbeet pulp were comparable. Pectin hydrolysis was found to be slightly increased using pressed pulp. Solid loading rates can be increased up to 8% with little or no impact on hydrolysis. Further increases in solids loading rate may be limited by mixing requirements but would increase sugar and ethanol concentrations leading to lower hydrolysis, fermentation, and distillation costs.

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