Process Yield and Economic Trade-offs for Enzymatic Hydrolysis of Alkaline Pretreated Corn Stover

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ABSTRACT

Response surface methodology was used to investigate the interaction of pH, temperature, and enzyme loadings on corn stover hydrolysis rates following soaking in aqueous ammonia pretreatment. Economic tradeoffs were estimated for cellulase and hemicellulase loadings under different hydrolysis conditions. Enzyme loadings had a more significant effect on rates than did pH or temperature. The effect of hydrolysis pH was independent of temperature and enzyme loadings, and the optimal pH for glucose and xylose yields were 4.5 and 4.3, respectively. Conducting hydrolysis at 50 °C rather than 37 °C enables either a 10% glucose yield increase, or a comparable yield with 40% and 65% reduction in cellulase and hemicellulase loadings, respectively. Although yield models showed that hydrolysis rates increase with higher enzyme loadings, economic models showed that optimal cellulase and hemicellulase loadings were as much as 47% and 23% lower, respectively, than the maximum loadings tested. Optimal enzyme loadings change with fluctuations in enzyme costs and ethanol price, but cellulase loadings were more sensitive to these changes than hemicellulase loadings. Enzyme loadings were also more sensitive to enzyme price at lower processing temperatures. Enzyme loadings can be adjusted to increase return based on enzyme costs, ethanol price, and process temperature.

Keywords Cellulase · Hemicellulase · Pretreatment · Enzymatic hydrolysis · Economic analysis

1. Introduction

The enzymatic hydrolysis of cellulosic biomass for producing industrial sugars is a promising strategy for efficient utilization of renewable resources. Cellulase enzymes are the most important for effective enzymatic hydrolysis. Other supplementary enzymes may also be required to aid the hydrolysis depending on the pretreatment technology [1, 2]. However, cost reduction by lowering enzyme loading will be critical to make bioindustries more competitive with petrochemical industries [3]. Though enzymes play a key role in the hydrolysis process, other factors such as pH and temperature also affect the overall yields. Therefore, developing a model to demonstrate the interactions of enzymatic hydrolysis factors to improve hydrolysis efficiency will have significant economic benefits.

Factors that affect enzymatic hydrolysis of cellulosic biomass includes the amount and type of substrate, enzyme loadings, and reaction conditions (e.g. pH and temperature). The range of pH for commercially available enzymes varies from 2.5 to 6.5, but enzymatic hydrolysis is typically carried out at a pH of 4.8 to 5.0 [4]. The optimal hydrolysis temperature varies from 45 to 70 °C depending on the enzyme mixture, but the most commonly used temperature is near 50 °C [4, 5]. Enzymatic hydrolysis conditions vary with process configurations such as separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). The temperature for enzymatic hydrolysis can be optimized independently from the fermentation temperature in SHF, whereas compromise is needed for an optimal process temperature in SSF. The use of 37 °C is a favorable condition for SSF, since optimal temperatures for the yeast *Saccharomyces cerevisiae* are usually near 30 °C, and enzymes from *T. reesei* have highest activity at around 45 to 50 °C [6]. However, there have been few reports of SSF using other strains or organisms with temperature and pH optima closer to those of commercial cellulases.

SSF at elevated temperatures were reported for ethanol production using *Candida acidothermophilum* and *Saccharomyces uvarum* [7]. Knowing how hydrolysis proceeds across the range of temperatures and pHs can allow mapping of those yields to microbial growth conditions for SSF processes with new organisms.

The primary objective of this study is to model how optimal cellulase and hemicellulase loadings change under a range of hydrolysis conditions (temperature and pH) after alkaline pretreatment. However, processes designed to optimize yield may not generate the greatest economic return. Consequently, we include an economic analysis that combines research-based yield models with select input and output market prices to estimate the most economically efficient processing conditions to compare with conditions based on yield optimization alone. The secondary objective was to determine potential economic tradeoffs between cellulase and hemicellulase enzyme loadings at different hydrolysis conditions.

2. Materials and methods

2.1. Raw material

Corn (*Zea mays L.*) stover (stalks and leaves) was collected from a USDA-ARS research field (46° 48' 38.51" N, 100° 54' 52.53" W) in Mandan, North Dakota, USA. Corn stover was air dried (10% moisture content, dry basis) and ground in a Wiley Mill with a 6 mm sieve. Sieved corn stover was stored in a sealed plastic bag at room temperature until use.

2.2. Soaking in aqueous ammonia (SAA) pretreatment

Biomass was pretreated by soaking in aqueous ammonia with 15% ammonium hydroxide mass fraction at 40 °C for 24 h. Ground corn stover was pretreated at a solid loading of 100 g L⁻¹ in a 2-L, screw-capped Pyrex bottle. The pretreatment bottle was placed in a preheated incubator at 40 °C for 24 h after the mixture reached the desired temperature. The pretreated

solids were separated by filtering through Whatman # 41 filter paper (20–25 μm pore size) using a vacuum filtration unit. The solids were washed with distilled water ($\sim 4\text{ L}$), weighed, and stored in sealed plastic bags at $4\text{ }^{\circ}\text{C}$ to use for subsequent enzymatic hydrolysis experiments. The moisture content and solid recovery of pretreated biomass were determined in triplicate by drying a small portion of wet solids ($\sim 2\text{ g}$) overnight in a convection oven at $105\text{ }^{\circ}\text{C}$. A portion of pretreated wet solids ($\sim 20\text{ g}$) was also dried at room temperature for compositional analysis.

2.3. Compositional analysis for carbohydrate and lignin determination

Total solids content, structural carbohydrate, and lignin content of non-pretreated raw corn stover and SAA- pretreated corn stover were determined using the standard National Renewable Energy Laboratory Analytical Procedures [8]. It is necessary to remove the non-structural carbohydrates such as nitrites/nitrates, proteins, chlorophyll and waxes from the biomass prior to structural carbohydrate analysis to prevent interference with downstream processing. The extractives were removed from the non-pretreated biomass using accelerated solvent extraction following the National Renewable Energy Laboratory (NREL) Chemical Analysis and Testing Procedures [9]. All compositional analysis was done in duplicate.

2.4. Enzymes

In order to investigate the influence of enzyme concentration on the enzymatic hydrolysis of SAA-pretreated corn stover, commercial enzyme solutions, NS50013 (cellulase complex), and Cellic HTec (hemicellulase) were used. A supplementary cellobiase (β -glucosidase) enzyme, Novozyme 188, was also used as cellulase from *T. reesei* is deficient in cellobiase, restricting the conversion of cellobiose to glucose. All enzymes were provided by Novozymes North America, Inc. (Franklinton, NC, USA). According to the manufacturer information sheet, the optimum temperature for cellulase complex NS50013 is in the range of ($45\text{--}50\text{ }^{\circ}\text{C}$), β -

glucosidase Novozyme 188 in the range of 45–70 °C and xylanase Cellic HTec operating temperature in the range of 45–50 °C. Regarding operating pH, the range for NS50013 is from 4.5 to 6.5, Novozyme 188 is from 2.5–6.5, and Cellic HTec operating pH is from 4.5–6.0. The cellulase activity of NS50013 and β -glucosidase activity of Novozyme 188, were 77 filter paper units (FPU) cm^{-3} and 500 cellobiase units (CBU) cm^{-3} , respectively, as determined by Ghose [10]. Xylanase activity of Cellic HTec, as determined by Bailey et al. [11] was 10,600 xylanase units (XU) cm^{-3} . No detectable cellulase activity was identified for Novozyme 188 or Cellic HTec. Cellobiase activity of NS50013 was 6 CBU cm^{-3} , which only contributed 0.07 CBU per FPU and thus considered negligible in calculations.

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis was carried out with SAA-pretreated corn stover in 0.125 L Erlenmeyer flasks (working volume of 0.05 L) with a 10 g L^{-1} glucan loading. This low loading was chosen to minimize enzyme product inhibition following the National Renewable Energy Laboratory (NREL) Analytical Procedures [12]. Hydrolysis pH was adjusted between 4 and 5.4 with sodium citrate buffer (50 mol m^{-3}) according to the experimental design. Cellulase and hemicellulase were added on glucan mass basis according to the experimental design. β -glucosidase was added at a constant ratio of cellulase (FPU): β -glucosidase (CBU) at 1:1 across all treatments. Sodium azide was added to the mixture at a concentration of 400 $\mu\text{g L}^{-1}$ to prevent microbial contamination during enzymatic hydrolysis. Flasks were placed in a water bath shaker (MaxQ 7000, Thermo Scientific, Dubuque, IA, USA) and temperature was varied (37–50 °C) based on the experimental design. All flasks were continuously agitated at 2.17 Hz for 72 h. Aliquots (1 cm^3) were taken at 24 h intervals from each flask and immediately centrifuged at $13,226 \times g$ for 5 min (Galaxy 16 Micro-centrifuge, VWR International, Bristol, CT, USA). After

centrifugation, the supernatant was filtered through a 0.2 μm nylon filter (Pall Corporation; West Chester, PA, USA) and stored at $-20\text{ }^{\circ}\text{C}$ until sugar analysis.

2.6. High performance liquid chromatography (HPLC) analysis

Hydrolysis samples were analyzed by HPLC (Waters Corporation; Milford, MA) equipped with an autosampler, an isocratic pump, and a refractive index (RI) detector (model 2414, Waters Corporation). The sugars were analyzed using a Bio-Rad Aminex HPX-87P ($300 \times 7.8\text{ mm}$) carbohydrate column (Bio-Rad Laboratories; Hercules, CA) and quantified with column and detector temperatures of $85\text{ }^{\circ}\text{C}$ and $50\text{ }^{\circ}\text{C}$, respectively. The sugars from the injected sample (20 mm^3) were eluted with $18\text{ m}\Omega$ NANOpure water at a flow rate of $0.6\text{ cm}^3\text{ min}^{-1}$. Glucose and xylose were quantified using 4-point external standard curves with mixtures of cellobiose, glucose, xylose, galactose, and arabinose to quantify sugar concentrations in the sample.

2.7. Experimental design and statistical analysis

Response surface modeling (RSM) was used to model the effect of pH, temperature, cellulase and hemicellulase enzyme loadings on SAA- pretreated (15% ammonia at $40\text{ }^{\circ}\text{C}$ for 24 h) corn stover hydrolysis yield using a Box-Behnken design of four variables and three levels as defined in Table 1. The ranges of independent variables for cellulase and hemicellulase enzyme were selected based on the results of preliminary experiments (not shown). The lower level of temperature was chosen at $37\text{ }^{\circ}\text{C}$, typical of the SSF process while the highest level at $50\text{ }^{\circ}\text{C}$, representing typical optimal for SHF. pH range was selected between 4 and 5.4 as this range was reported to be optimal for all three enzymes [13]. The complete design consisted of 27 treatments, including three repetitions at the center point (Table 2).

Table 1

Glucose and xylose yields (% of theoretical) at 24 h of hydrolysis were chosen as representation of hydrolysis rate. The experimental data were analyzed by the response surface regression procedure using the following quadratic polynomial model (Equation 1).

$$R = \beta_0 + \beta_P P + \beta_T T + \beta_C C + \beta_H H + \beta_{PP} P^2 + \beta_{TT} T^2 + \beta_{CC} C^2 + \beta_{HH} H^2 + \beta_{PT} PT + \beta_{PC} PC + \beta_{PH} PH + \beta_{TC} TC + \beta_{TH} TH + \beta_{CH} CH \quad \text{Equation (1)}$$

R is the response variable (glucose or xylose yield, % of theoretical). P , T , C , and H represent the uncoded independent variables for pH, temperature ($^{\circ}\text{C}$), cellulase (FPU g^{-1} glucan) and hemicellulase (XU g^{-1} glucan), respectively. The terms β_0 , β_i , β_{ii} , and β_{ij} are the constant, linear, quadratic and interaction model coefficients, respectively. Statistically insignificant ($p > 0.05$) terms were identified and removed sequentially until all remaining terms were significant to develop quadratic polynomial regression equations and resulting models. Their significance was evaluated by analysis of variance (ANOVA). The fit of the model was evaluated by the determination of R-squared coefficient, adjusted R-squared coefficient, and lack-of-fit test. Each variable accounts for a certain amount of the total variance of the response variable, and so the relative contributions can be quantified for each. The percentage of contribution for each term was estimated by dividing the sum of squares (SS) for that term by total SS. The final relationship is represented as contour plots in order to visualize the relationship between the response and experimental levels of each factor. MINITAB 17 statistical software (Minitab Inc.; State College, PA) was used to design the experiment, to develop the empirical model, and to generate the response surface plots.

Table 2

2.8. Determining the economically optimal loadings of cellulase and hemicellulase

Finding the optimal loadings of enzymes for producing fermentable sugars (glucose and xylose) by SSF and SHF is important to increase the economic efficiency. We use a common profit maximization framework from the field of microeconomic analysis to compare the economic impacts of enzyme loadings of the two processes [14]. The analysis compares the economic tradeoffs for cellulase and hemicellulase since a range of loadings can result in equivalent hydrolysis rates. Results from bench scale experiments in this study are used to estimate the economic optimal loadings of cellulase and hemicellulase.

We assume the biorefinery is a profit maximizer and price taker in the input and product markets. Consequently, the biorefinery chooses levels of inputs (X_j), and outputs (Y_i) that maximize profits (π) given the prices of inputs (P_j) and outputs (P_i):

$$MAX \pi = \sum_{i=1}^n P_i Y_i - \sum_{j=1}^m P_j X_j \quad \text{Equation (2)}$$

The biorefinery is assumed to be operating in the short run, so the use of at least one input, capital, and its associated costs are fixed. Specifically, we assume that the biorefinery is optimizing enzyme loadings for a facility that is already outfitted with required preprocessing and other capital equipment. Our economic analysis focuses on cellulase (c) and hemicellulase (h) enzymes. We do not include β -glucosidase explicitly but it could be modeled as an integral part of cellulase costs. Glucose and xylose are intermediate products that are fermented into ethanol, representing the biorefinery's primary product.

The biorefineries short-run profit maximizing equation is

$$MAX \pi = P_e Q_e(\text{glucose}(h, c), \text{xylose}(h, c)) - P_c Q_c - P_h Q_h \quad \text{Equation (3)}$$

Optimal use of variable inputs can be found by solving the first order conditions:

$$P_c = P_e \left(\frac{\partial Q_e}{\partial \text{glucose}} \frac{\partial \text{glucose}(c, h)}{\partial c} + \frac{\partial Q_e}{\partial \text{xylose}} \frac{\partial \text{xylose}(c, h)}{\partial c} \right) \quad \text{Equation (4)}$$

$$P_h = P_e \left(\frac{\partial Q_e}{\partial \text{glucose}} \frac{\partial \text{glucose}(c,h)}{\partial h} + \frac{\partial Q_e}{\partial \text{xylose}} \frac{\partial \text{xylose}(c,h)}{\partial h} \right) \quad \text{Equation (5)}$$

Where P_c , P_h , and P_e represent prices of cellulase, hemicellulase and ethanol, and Q_c , Q_h , and Q_e represent the quantities of cellulase, hemicellulase and ethanol, respectively. These equations are the variable input profit maximizing conditions: marginal revenue product equals marginal input cost for cellulase and hemicellulase. Conversion efficiencies of glucose to ethanol (95%), xylose to ethanol (85%), ethanol stoichiometric yield (51%), and ethanol price of \$0.634 L⁻¹ are taken from Humbird et al. [15]. The common enzyme price is assumed at \$4.70 kg⁻¹ protein according to Hong et al. [16].

3. Results and discussion

3.1. Composition of non-pretreated and SAA- pretreated corn stover and hydrolysis yields

The chemical composition of non-pretreated corn stover and corn stover pretreated by SAA are summarized in Table 3. Nearly 70% of solid was recovered after SAA pretreatment. The majority of glucan (82%) and xylan (72%) were preserved in the pretreated solids after SAA pretreatment. Solids loss during this pretreatment was mainly attributed to lignin removal (55%). The solid retention, carbohydrate retention and lignin removal are in agreement with data in previous reports obtained for SAA pretreated switchgrass [17, 18], and corn stover [19].

Table 3

Table 2 shows the experimental conditions and resulting 24-h hydrolysate concentrations and yields, according to the design. Glucose and xylose yields at 24 h, expressed as a percent of the theoretical, were chosen for the response surface modeling because the intermediate yield is more representative of initial hydrolysis rates than ultimate hydrolysis yields at 48 or 72 h. By

applying multiple regression analysis on the experimental data, the response variable and the test variables were related by a second-order polynomial equation.

The final models of glucose and xylose yields at 24 h (% of theoretical), after removal of nonsignificant terms, are represented by Equations (6) and (7), respectively.

$$\begin{aligned} \textbf{Glucose model: } R = & -14.8 + 29.3(P) - 0.524(T) + 0.392(C) + 2.25 \times 10^{-2}(H) - 3.29(P)^2 - \\ & 1.57 \times 10^{-2}(C)^2 - 0.019 \times 10^{-3}(H)^2 + 2.40 \times 10^{-2}(TC) + 4.15 \times 10^{-4}(TH) - 4.08 \times 10^{-4}(CH) \end{aligned} \quad \text{Equation (6)}$$

$$\begin{aligned} \textbf{Xylose model: } R = & -190 + 56.1(P) + 4.29(T) + 1.56(C) + 3.39 \times 10^{-2}(H) - 6.50(P)^2 - 4.66 \times 10^{-2}(T)^2 - \\ & 1.92 \times 10^{-2}(C)^2 - 0.16 \times 10^{-4}(H)^2 - 3.90 \times 10^{-4}(CH) \end{aligned} \quad \text{Equation (7)}$$

The results were analyzed using analysis of variance (ANOVA) and response plots. The statistical significance of the ratio of the mean square variation due to regression and mean square residual error was tested using ANOVA. Corresponding ANOVA tables for both models are shown in Table 4. For glucose yield, the model fit the data very well with an R^2 , adjusted R^2 , and predicted R^2 value of 0.977, 0.963, and 0.924, respectively. The lack of fit of the model was not significant (p -value = 0.169), further indicating model adequacy. For xylose yield, the model fit the data very well with an R^2 , adjusted R^2 , and predicted R^2 value of 0.972, 0.957 and 0.923, respectively. A lack-of-fit value of 0.353 also supports the fitness of the model. Fig. 1 shows the relationship of both glucose and xylose yield calculated by the model plotted against the corresponding experimental data, which further illustrates the model adequacy over the full range of the data.

Figure 1

The ANOVA shows the percent contribution of linear, quadratic and interaction terms of four independent variables, namely pH, temperature, cellulase and hemicellulase (Table 4). For glucose yield, linear terms account for the majority of the variation (83%), whereas quadratic and interaction terms account for 12% and 3%, respectively. As can be seen from the ANOVA table,

among the independent variables cellulase and hemicellulase enzymes had a higher significant effect ($p < 0.001$) than the pH and temperature ($p < 0.05$).

For the xylose model, linear terms accounted most of the variability (86%) followed by around 10% variability from quadratic terms and only 1.4% contribution from the interaction between variables. Among all the variables in xylose model, the cellulase and hemicellulase enzyme together contributed approximately 80% of the variation.

Table 4

The variance inflation factor (VIF) is used to check how much multicollinearity (correlation between predictors) exists in the regression as this could be problematic making the regression coefficient unstable and difficult to interpret. When significant multicollinearity issues exist, the VIF will be very large (5 to 10) for the variables involved. In terms of VIF for both glucose and xylose model, all the linear and interaction terms resulted in a VIF of 1 while the quadratic terms had a VIF of 1.11 and 1.25, respectively for glucose and xylose model indicating no correlation among the predictors. Fig. 2 shows the profiles for predicting (a) glucose and (b) xylose 24-h yields and the variation of each main variables as there is not much interaction among the variables. Our result shows that variation in enzyme loading is more important than pH or temperature fluctuation, especially for glucose yields.

Figure 2

The predicted optimal pH for glucose yield was 4.5, which is lower than most commonly used pH values (4.8) for commercial cellulase use. Using lower optimal pH for commercial use of cellulase might get benefit to reduce microbial contamination at lower pH values. The highest glucose yield was achieved at the highest temperature tested, 50 °C, and temperature had greater impact than pH (Fig. 2–a). This finding is contrary to another study which found that the change

in temperature was less important than the change in pH [20] when optimizing parameters for thermostable cellulase activity from *Aspergillus heteromorphus*, indicating difference in response based on the enzyme-producing organisms.

The optimal pH and temperature for xylose yields are lower than for optimal glucose yields. The predicted optimal pH for xylose yield was 4.3 and the optimal temperature was found 46 °C. Fig. 2b shows that xylose yields decrease more rapidly than glucose yields with increasing pH, indicating that the hemicellulase enzymes used are more sensitive to pH changes.

Optimal pH values for both glucose and xylose yields in this study were lower than reported in other studies. Our study shows no interaction between pH and temperature for glucose or xylose yields of SAA-pretreated corn stover. The absence of interaction between these variables indicates an additive effect of these variables on the response. Pandiyan et al. [21] reported a minimal effect of pH from 4 to 5 on the saccharification yield of alkali pretreated *Parthenium* sp. but also found no significant interaction between pH and temperature. On the contrary, Wang et al. [22] reported pH values greater than 5.5 enhanced saccharification of SPORL (Sulfite Pretreatment to Overcome Recalcitrance of Lignocelluloses) pretreated lodgepole pine when enzymatic hydrolysis was conducted at 50 °C with Celluclast 1.5 L. Differences in the best enzymatic hydrolysis condition is attributed to the variation in the chemical composition of the different biomass, type of pretreatment, as well as the enzyme formulation used. The lack of interaction between temperature and pH is more likely to hold across substrates and pretreatments.

Fig. 2 shows that the temperature relationship is nearly linear for 24-h glucose yields. Increasing both glucose and xylose yields at higher temperature shows the practicality of developing other microorganism strains that can tolerate higher temperatures than

Saccharomyces cerevisiae. Several studies have focused on development of thermotolerant microbes for SSF conditions for producing ethanol, butanol or other biobased chemicals. Using an engineered strain of *Thermoanaerobacterium saccharolyticum* ALK2 for SSF at 50 °C, Shaw et al. [23] showed 40% enzyme loading reduction over SSF using *S. cerevisiae* at 37 °C. Kadam and Schmidt [24] found a thermotolerant yeast, *Candida acidotherophilum*, produced 80% of the theoretical ethanol yield at 40 °C using dilute acid pretreated poplar as substrate. Another strain, *Kluyveromyces marxianus* identified by Ballesteros et al. [25] have been reported as more thermotolerant (42 °C) than *Candida* and *Saccharomyces* strains. These strains need to be tested to find efficiency in SSF conditions with different pretreated biomass. Any improvement in strain robustness at higher temperatures will have a direct impact on hydrolysis rates. Even though this information is not new, most studies only show relationship with one enzyme and temperature. This study demonstrates the effect with combined enzymes with any pH between 4.0 and 5.5.

Contour plots were developed to illustrate the interactive effects of different combinations of enzyme loadings, where pH and temperature were kept constant. Such plots can be developed for any combination of pH and temperature based on the desired conditions, but we illustrated the differences using typical SSF and SHF temperatures with optimal pH values (Fig. 3). The plots of glucose yield shows that temperature has a somewhat larger impact at higher enzyme loadings than lower loadings. This differential impact is due to the significant interaction terms for temperature and enzyme loadings (Table 4). These interactions are not present for xylose models. As we chose 24-h yields for modeling, these yields are more representative of initial hydrolysis rates rather than ultimate yields.

Figure 3

Although it is expected that higher enzyme loadings result in higher hydrolysis rates, this work demonstrates that different combinations of enzymes can be used to achieve similar results. The law of diminishing returns suggests that economically optimal loadings may not be loadings that result in the absolute highest rates or yields. To demonstrate those trade-offs within the design space, we looked at how much each enzyme loading could change under typical SSF (37 °C) and SHF (50 °C) conditions while still predicting 24-h glucose yield of 95% of the predicted optimal value (Fig. 4).

Figure 4

In typical SSF conditions, the predicted optimal loadings of cellulase and hemicellulase at 30 FPU and 720 XU g⁻¹ glucan respectively, resulted in a predicted glucose yield of 64% at 24 h. Our glucose model predicts that cellulase loadings can be reduced as much as 43%, equivalent to 17 FPU g⁻¹ glucan, and still maintain 95% of the predicted optimal values with hemicellulase loadings of 720 XU g⁻¹ glucan. On the contrary, hemicellulase loadings can be reduced as much as 62% from the predicted optimum and maintain 95% of the predicted optimal values with cellulase loadings of 30 FPU g⁻¹ glucan (Fig. 4).

For SHF conditions, the predicted optimal loadings of cellulase and hemicellulase were 30 FPU and 780 XU g⁻¹ glucan for maximum glucose yield (around 71%). To achieve 95% of optimal prediction which is 67% of glucose yield at 24 h, cellulase loadings can be reduced to 26% (22 FPU g⁻¹ glucan) with the higher hemicellulase loadings (780 XU g⁻¹ glucan). In contrast, hemicellulase loadings can be reduced by 51% (380 XU g⁻¹ glucan), with high cellulase loadings (30 FPU g⁻¹ glucan). The graphs of 24-h xylose yields for varying cellulase and

hemicellulase enzymes at typical SSF and SHF temperatures are not shown as they followed a similar trend to glucose yield.

Enzyme loadings have been reported as the most important factor after effective pretreatment for increasing the rate or yield of enzymatic hydrolysis [26, 27]. Our models show that glucose and xylose are similarly sensitive to cellulase and hemicellulase for SAA pretreated corn stover. Gupta et al. [28] previously explained that hemicellulase supplementation not only increases the xylan digestibility of treated biomass but also the glucan digestibility by reducing the hindrance caused by a resilient hemicellulose layer on the cellulose microfibrils. As cellulase loading increases, the effect of hemicellulase becomes less important but never negligible (Fig. 3). This study highlights the need for both enzymes after SAA pretreatment and shows how they can be used in a complementary way. Further, based on market costs of each enzyme, loadings can be adjusted to minimize cost while maintaining higher hydrolysis rates.

3.2. Economic analysis

An economic analysis was conducted for SAA-pretreated corn stover assuming typical pH and temperature for SSF and SHF processes. Optimal enzyme loadings to maximize hydrolysis rates or yields may not coincide with optimal loadings to maximize economic return. Table 5 shows the economically optimal enzyme loadings based on enzyme costs and ethanol revenues.

Table 5

The baseline economically optimal loadings of cellulase and hemicellulase were 38% and 5% higher, respectively, for SHF than for typical SSF temperatures. The higher optimal loadings for SHF are somewhat counterintuitive. We would expect that the SHF process would require less enzyme than for typical SSF conditions because hydrolysis rates are higher under SHF

conditions. The yield model predicted 24-h glucose yields of 60.5% for typical SSF temperatures, and 67.1% for SHF, for the optimal enzyme loadings, whereas maximum 24-h yield in the design space were 64% and 71%, respectively. Although higher yields would be expected for longer hydrolysis times, differences between treatments would be more difficult to distinguish and the 24-h yields are more representative of initial hydrolysis rates. The basic economic model suggests that the higher ethanol revenue that comes with SHF outweighs the associated cost of higher enzyme loadings. The models show that there is a point of diminishing returns on enzyme loadings for process temperatures, but that point is reached at lower cellulase and hemicellulase loadings for the lower temperatures typically required for SSF.

Enzyme prices directly impact the economically optimal loadings, but estimating realistic enzyme prices is challenging. Publicly available information is limited due to the proprietary nature of data from enzyme manufacturers [29]. Enzyme cost estimates also vary based on ongoing enzyme development, different enzyme source microorganisms, and mixtures of enzymes activities within a commercial product [15, 16]. We addressed the situation by conducting a sensitivity analysis on each enzyme price. Economic optimum enzyme loadings were estimated while adjusting enzyme prices 20% higher or lower than the assumed baseline prices, and these results are also shown in Table 5.

In comparing typical SHF and SSF processing temperatures, optimal enzyme loadings are more sensitive to enzyme price fluctuations for typical lower temperature SSF processes than higher temperature SHF processes. Optimal cellulase and hemicellulase loadings for both processes are more sensitive to cellulase cost than hemicellulase cost (Table 5). Hemicellulase cost changes of 20% had little impact (<1%–6%) on optimal loadings for either enzyme. However, 20% variation in cellulase price caused changes of up to 25% in cellulase loadings.

Ethanol selling price also varies considerably due to market fluctuations. An ethanol price of \$0.634 L⁻¹ (\$2.20 gallon⁻¹) was selected for the baseline scenario [15]. Given price uncertainty, we also looked at how sensitive the optimum enzyme loadings are by varying ethanol price by 20%. Fluctuations in ethanol price have a greater impact on economically optimal cellulase loadings than on hemicellulase loadings. Effects of ethanol price changes are similar in magnitude to the effects of cellulase cost changes. However, increasing cellulase costs decreases optimal enzyme loadings, while ethanol price increases allow the biorefinery to further increase both yields and profits by adding more enzyme. Similarly, when ethanol prices drop, so does the point of diminishing returns on enzyme loadings and the lower revenue requires a reduction in enzyme loadings.

4. Conclusions

The effect of pH on hydrolysis rates is independent of temperature or enzyme loadings. Models show that glucose and xylose rates were highest at pH values of 4.5 and 4.3, respectively, lower than those typically reported in the literature. Changes in temperature and pH have a much lower effect on 24-h glucose yields than on xylose yields. Use of higher temperature SHF conditions leads to higher hydrolysis rates but necessitates higher enzyme loadings. However, similar rates are possible in SHF with lower enzyme loadings when compared to the lower temperatures for typical SSF conditions. This work shows the interaction between cellulase and hemicellulase loadings on hydrolysis rates and demonstrates that higher loadings of one enzyme can compensate for lower loadings of the other. Therefore, many combinations of enzyme loadings can be used to achieve similar hydrolysis rates. Optimal enzyme loadings to maximize economic return are lower than loadings to maximize rates.

Cellulase costs and ethanol price both have a larger effect than hemicellulase costs in determining loadings optimized for economic return.

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Figure Captions

Fig. 1. Scatter diagram of predicted response versus actual response of hydrolysis yields after 24 h hydrolysis of SAA-pretreated corn stover.

Fig. 2. Profiles for predicting (a) glucose and (b) xylose yields for 24 h hydrolysis for main effects of pH, temperature, cellulase, and hemicellulase loadings for SAA-pretreated corn stover. Each line represents the mean response for the given main factor across all modeled conditions for the remaining factors.

Fig. 3. Contour plots of the combined effects of cellulase and hemicellulase on glucose 24-hr yields at (a) typical SSF (37 °C) and (b) SHF condition (50 °C) using SAA-pretreated corn stover with 15% aqueous ammonia for 24 h at 40 °C.

Fig. 4. Cellulase and hemicellulase tradeoffs for predicting glucose yield of 95% of the predicted maximum for typical SSF (simultaneous saccharification and fermentation) and SHF (separate hydrolysis and fermentation) temperatures while pH held constant at 4.5.

Table 1 Independent process variables and their levels used in the Box-Behnken experimental design for the SAA-pretreated (15% ammonia at 40 °C, 24 h) corn stover hydrolysis.

Independent variables	Symbol	Factor level		
		Low (-1)	Middle (0)	High (+1)
pH	<i>P</i>	4	4.7	5.4
Temperature (° C)	<i>T</i>	37	43.5	50
Cellulase (FPU g ⁻¹ glucan)	<i>C</i>	10	20	30
Hemicellulase (XU g ⁻¹ glucan)	<i>H</i>	0	500	1000

Table 2 Response surface Box-Behnken design and resulting glucose and xylose yields after 24 h enzymatic hydrolysis of SAA-pretreated corn stover with 15% ammonia at 40 °C for 24 h.

Run	Design uncoded factors (actual levels)				Concentration		Yield (% of theoretical)			
	pH	Temp.	Cellulase	Hemicellulase	Glucose	Xylose	Glucose		Xylose	
		(°C)	(FPU g ⁻¹ glucan)	(XU g ⁻¹ glucan)	(g L ⁻¹)	(g L ⁻¹)	Actual	Predicted	Actual	Predicted
1	4	37	20	500	6.8	3.5	61.0	59.9	58.4	57.2
2	5.4	37	20	500	6.5	3.0	58.7	57.6	51.0	50.2
3	4	50	20	500	6.7	3.4	59.9	62.1	58.2	60.3
4	5.4	50	20	500	6.8	3.2	61.3	59.8	54.5	53.3
5	4.7	43.5	10	0	4.6	2.5	41.5	40.4	41.5	41.5
6	4.7	43.5	30	0	6.5	3.5	58.1	56.5	58.8	57.3
7	4.7	43.5	10	1000	6.4	3.2	57.4	57.9	54.8	55.6
8	4.7	43.5	30	1000	7.3	3.8	66.0	65.8	64.3	63.6
9	4	43.5	20	0	5.5	3.1	49.4	49.5	51.8	51.6
10	5.4	43.5	20	0	5.1	2.6	46.3	47.3	43.9	44.6
11	4	43.5	20	1000	7.1	3.7	63.8	63.0	62.2	61.8
12	5.4	43.5	20	1000	6.6	3.2	59.3	60.7	53.5	54.8
13	4.7	37	10	500	5.9	2.8	52.7	54.4	48.0	49.0
14	4.7	50	10	500	5.9	3.1	53.4	53.4	52.2	52.1
15	4.7	37	30	500	6.9	3.5	62.1	63.3	59.2	60.9
16	4.7	50	30	500	7.7	3.8	69.0	68.6	64.8	64.0
17	4	43.5	10	500	6.0	3.1	53.6	53.4	53.2	52.8
18	5.4	43.5	10	500	5.7	2.7	51.0	51.1	46.5	45.8
19	4	43.5	30	500	7.2	3.8	64.5	65.4	64.1	64.8
20	5.4	43.5	30	500	6.9	3.3	62.0	63.2	56.5	57.8
21	4.7	37	20	0	5.5	2.8	49.2	50.3	48.0	47.8
22	4.7	50	20	0	5.4	2.9	48.2	49.8	48.4	50.9
23	4.7	37	20	1000	6.8	3.4	60.9	61.0	58.2	58.0
24	4.7	50	20	1000	7.3	3.7	65.3	65.9	62.6	61.1
25	4.7	43.5	20	500	6.8	3.5	61.0	61.5	59.5	60.4
26	4.7	43.5	20	500	6.9	3.6	62.2	61.5	61.3	60.4
27	4.7	43.5	20	500	6.8	3.5	61.6	61.5	60.0	60.4

Table 3 Chemical composition (dry basis) of non-pretreated and soaking in aqueous ammonia (SAA) pretreated corn stover.

	S.R. ¹ (%)	Glucan (%)	Xylan (%)	Lignin ² (%)
Non-pretreated corn stover	-	41.2 ± 3.83	21.3 ± 2.23	20.2 ± 0.220
SAA-pretreated corn stover	69.7	48.2 ± 0.820	21.7 ± 2.55	12.9 ± 2.23

¹ S.R.: Solid remaining after pretreatment; ² Sum of acid insoluble and soluble lignin

Table 4 ANOVA for response surface models of glucose and xylose yields (% of theoretical) after 24 h hydrolysis when varying hydrolysis pH, temperature and cellulase and hemicellulase enzyme loadings of SAA-pretreated corn stover with 15% ammonia at 40 °C for 24 h.

Source of variation	DF	Seq SS	Adj SS	Adj MS	F-value	<i>p</i> -value
Glucose Model						
Regression	10	1168.50	1168.50	116.85	68.93	0.001
Linear	4	996.88	996.88	249.22	147.01	0.001
pH (<i>P</i>)	1	15.41	15.41	15.41	9.09	0.008
Temperature (<i>T</i>)	1	13.44	13.44	13.44	7.93	0.012
Cellulase (<i>C</i>)	1	434.16	434.16	434.16	256.11	0.001
Hemicellulase (<i>H</i>)	1	533.87	533.87	533.86	314.93	0.001
Square	3	138.00	138.00	46.00	27.14	0.001
<i>P</i> × <i>P</i>	1	0.79	15.57	15.57	9.19	0.008
<i>C</i> × <i>C</i>	1	0.92	14.81	14.81	8.74	0.009
<i>H</i> × <i>H</i>	1	136.29	136.29	136.29	80.40	0.001
2-Way Interaction	3	33.61	33.61	11.20	6.61	0.004
<i>T</i> × <i>C</i>	1	9.70	9.70	9.70	5.72	0.029
<i>T</i> × <i>H</i>	1	7.26	7.26	7.26	4.28	0.055
<i>C</i> × <i>H</i>	1	16.65	16.65	16.64	9.82	0.006
Residual Error	16	27.12	27.12	1.69		
Lack of fit	14	26.41	26.41	1.88	5.31	0.169
Pure error	2	0.71	0.71	0.35		
Total	26	1195.62				
			$R^2 = 0.977$	$R^2_{\text{adj}} = 0.963$	$R^2_{\text{pred}} = 0.924$	
Xylose Model						
Regression	9	1050.80	1050.80	116.75	64.92	0.001
Linear	4	932.57	932.58	233.14	129.63	0.001
pH (<i>P</i>)	1	147.00	147.00	147.00	81.74	0.001
Temperature (<i>T</i>)	1	26.70	26.70	26.70	14.85	0.001
Cellulase (<i>C</i>)	1	426.02	426.02	426.02	236.88	0.001
Hemicellulase (<i>H</i>)	1	332.85	332.85	332.85	185.07	0.001
Square	4	103.01	103.01	25.75	14.32	0.001
<i>P</i> × <i>P</i>	1	17.57	54.05	54.04	30.05	0.001
<i>T</i> × <i>T</i>	1	1.69	20.72	20.71	11.52	0.003
<i>C</i> × <i>C</i>	1	2.29	19.68	19.67	10.94	0.004
<i>H</i> × <i>H</i>	1	81.47	81.47	81.46	45.30	0.001
2-Way Interaction	1	15.21	15.21	15.21	8.46	0.01
<i>C</i> × <i>H</i>	1	15.21	15.21	15.21	8.46	0.01
Error	17	30.57	30.57	1.79		
Lack of fit	15	28.85	28.85	1.92	2.23	0.353
Pure error	2	1.73	1.73	0.86		
Total	26	1081.37				
			$R^2 = 0.972$	$R^2_{\text{adj}} = 0.957$	$R^2_{\text{pred}} = 0.923$	

Table 5 Economic optimal loadings of cellulase (C) and hemicellulase (HC) at base case and with 20% changes from baseline for enzyme cost, and ethanol selling price (high + and low -) for typical SSF (simultaneous saccharification and fermentation) and SHF (separate hydrolysis and fermentation) process temperatures. Values in lower section of table represent percent change from the baseline scenario for each adjustment in enzyme or ethanol price.

Condition	Enzyme	Baseline	High Hemicellulase Cost	Low Hemicellulase Cost	High Cellulase Cost	Low Cellulase Cost	High Ethanol Price	Low Etahnol Price
SSF	C (FPU)	16	17	16	13	20	19	12
	HC (XU)	771	756	786	810	732	750	800
SHF	C (FPU)	22	22	22	19	26	25	18
	HC (XU)	807	793	822	846	769	790	840
SSF	C (FPU)	-	+ 6.3 %	0 %	- 19 %	+ 25 %	+ 19 %	- 25 %
	HC (XU)	-	- 2.0 %	+ 2.0 %	+ 5.1 %	- 5.1 %	- 2.7 %	+ 3.8 %
SHF	C (FPU)	-	0 %	0 %	- 14 %	+ 18 %	+ 14 %	- 18 %
	HC (XU)	-	- 1.7 %	+ 1.9 %	+ 4.8 %	- 4.7 %	- 2.1 %	+ 4.1 %