Title: β -glucosidase Supplementation during Biomass Hydrolysis: How Low Can We Go?

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22 **Abstract**

23 Corn stover was pretreated with dilute sulfuric acid to test the interaction effects of pH, cellulase
24 loading and β -glucosidase loadings on biomass hydrolysis rates. A response surface model was
25 developed showing that β -glucosidase supplementation had limited practical impact on
26 hydrolysis between 0.5 and 2 CBU FPU⁻¹. The only significant interaction in the model was
27 between pH and β -glucosidase loading but it also had little practical significance. Corn stover
28 and corn cob were used to test the effects of further β -glucosidase reduction. Significant
29 hydrolysis improvements were seen when 0.2 CBU FPU⁻¹ were added but very little
30 improvement was seen for higher loadings. Residual cellobiose concentrations confirmed these
31 findings but suggest that further reductions would have more noticeable negative effects on
32 hydrolysis rates. Results show that β -glucosidase supplementation during biomass hydrolysis can
33 be reduced to between 10 % and 20 % of typical loadings.

34 **Keywords**

35 β -glucosidase; Cellobiase; Cellulase; Cellobiose; Cellulose; Biomass hydrolysis

Introduction

Hydrolysis of biomass for production of fermentable sugars as a feedstock for fuels and chemicals is more of an economic challenge than a technical one. Costs for all operations from harvesting, storage, and transportation to pretreatment, hydrolysis and fermentation need to be minimized to increase bioindustry competitiveness with existing petrochemical industries. Relatively small efficiency improvements can result in significant economic savings when scaled up to facilities which could process more than 500,000 t of biomass annually.

One area of potential cost reduction is enzyme loading. Many researchers had calculated enzyme prices for cellulosic ethanol to be below 0.11 \$ L⁻¹ ethanol [1-3] while a more recent study resulted in cost models suggesting 0.18 \$ L⁻¹ ethanol under the best case scenario and between 0.30 \$ L⁻¹ and 0.39 \$ L⁻¹ ethanol when using more realistic processing assumptions of saccharification and fermentation yields [4].

Efficient biomass saccharification requires cellulase enzymes and, depending on pretreatment technology, supplementary enzymes to aid hydrolysis of hemicellulose and other residual components [5]. Supplementary β -glucosidase is also required to maximize hydrolysis rates and yields of glucose monomers. Typical commercial cellulase cocktails from *Trichoderma reesei* are deficient in β -glucosidase resulting in high residual concentrations of cellobiose, a known inhibitor of cellulase activity [6, 7].

Cellulase loadings are most often quantified based on the enzymatic activity, measured as filter paper units (FPU), per mass of cellulose or total input biomass. Goals for enzyme loadings are typically (10 to 20) FPU g⁻¹ cellulose following effective pretreatment [8-10] although

researchers regularly test loadings exceeding 30 FPU g⁻¹ [11-17]. β -glucosidase loadings are quantified as cellobiase units (CBU) and these loadings are often determined relative to the FPU loadings by setting a fixed CBU:FPU ratio. β -glucosidase loadings in the literature vary significantly. Although β -glucosidase loadings of 2 CBU FPU⁻¹ [16, 18, 19] appear to be the most common, others have used loadings as low 0.5 CBU FPU⁻¹ [20-22] and as high as 4 CBU FPU⁻¹ [23, 24]. A Plackett-Burman design used in another study showed that β -glucosidase did not have a significant effect on glucose yields during biomass hydrolysis but this study used cellulases derived from [25].

Enzymatic hydrolysis of biomass is typically carried out at a pH of 4.8 to 5.0, although recent studies with *T. reesei* cellulase complexes have suggested that higher hydrolysis rates could be realized at elevated pH values as high as 6.2 [26, 27]. Other studies using cellulases from *T. reesei* have resulted in empirical models suggesting that the optimum hydrolysis pH is between 4.5 and 4.9 [25, 28].

The initial objective of the research was to quantify the potential interaction of hydrolysis pH, cellulase loading, and β -glucosidase loading on hydrolysis rates using dilute acid-pretreated corn stover. The objective of follow-up experiments was to determine the extent to which β -glucosidase loading could be reduced below the range typically cited in the literature.

Materials and Methods

Corn stover (*Zea mays* L.) was collected from a USDA-ARS research field (46°48' 38.51"N 100°54' 52.53"W) in Mandan, North Dakota, USA. Corn cobs were collected from a North Dakota Agricultural Experiment Station farm (46°53' 42.75"N 96°48' 42.52"W) in Fargo, North

Dakota, USA. Corn stover and corn cob were stored at 4.5 % and 4.9 % moisture content (wet basis), respectively, and ground in a Wiley Mill with a 2-millimeter sieve. Composition of initial and pretreated materials was determined using NREL standard protocols for laboratory analysis [29] and are shown in Table 1.

Table 1

Biomass was pretreated using a dilute sulfuric acid treatment at 1.5 % H₂SO₄ at 140 °C for 20 min after reaching the desired temperature. Materials were pretreated at a solid loading of 0.1 g L⁻¹ in a 1-liter pressure vessel (4600 Series- Parr Industries; Moline, Ill). Pretreatment was stopped by immersing the reactor in an ice-water bath until reactor pressure reduced to 101.3 kPa. Pretreated biomass was filtered in a Buchner funnel under vacuum while washing with deionized water. Filtered solids were used for enzymatic hydrolysis.

Enzymatic hydrolysis was done using NS50013 as a (*T. reesei*) cellulase cocktail and Novozyme 188 as a β -glucosidase supplement. Enzyme activity was measured according to Ghose [30] as 66 filter paper units (FPU) cm⁻³ for NS50013 and 476 cellobiase units (CBU) cm⁻³ for Novozyme 188. Cellobiase activity of NS50013 was measured at 6 CBU cm⁻³ and considered negligible compared to supplementary levels of β -glucosidase used. The FPU is defined as the amount of enzyme which releases 2.0 mg of reducing sugar from 50 mg of Whatman No.1 filter paper in 1 h under the stated standard hydrolysis conditions [30, 31]. Likewise, the CBU is defined as the amount of enzyme which converts 1 μ mol of cellobiose to 2 μ mol of glucose per minute under standard assay conditions [30].

Hydrolysis pH was adjusted between 4.0 and 5.4 using 50 mol m⁻³ sodium citrate buffer. Sodium azide was added at a concentration of 4 x 10⁻⁴ g L⁻¹ to prevent microbial growth. Hydrolysis was carried out in 0.125-L Erlenmeyer flasks (working volume of 0.05 L) with a 0.01 g L⁻¹ glucan loading in a water bath shaker (MaxQ 7000, Thermo Scientific, Dubuque, IA, USA) at 50 °C stirred at 2.17 Hz for 72 h. Samples (1 cm³) were taken at 24-hour intervals and centrifuged with a relative centrifugal force of 13,226 × g for 5 min (Galaxy 16 Micro-centrifuge, VWR International, Bristol, CT, USA) before filtration through a nylon filter (Pall Corporation; West Chester, PA, USA) with a pore size of 0.2 µm. Samples were stored frozen at -20 °C until analysis.

Glucose and cellobiose concentrations of hydrolyzate samples were analyzed by HPLC (Waters Corporation; Milford, MA). The sample injection volume was 20 mm³ and 18 mΩ NANOpure water was used as mobile phase at a flow rate of 0.6 cm³ min⁻¹. A Bio-Rad Aminex HPX-87P column (Bio-Rad Laboratories; Hercules, CA) and a refractive index (RI) detector (model 2414, Waters Corporation) were used for analysis with column and detector temperatures of 85 °C and 50 °C, respectively. Quantification was done with four-point external standard curves using mixed carbohydrate standards.

Initial response surface modelling of the effects of hydrolysis pH, cellulase loading, and β-glucosidase loading was done using a Box-Behnken design and factor levels as defined in Table 2. Glucose concentrations after 24 h of hydrolysis were used as the response variable for modelling. Experimental design, model development, and response surface plots were created with MINITAB statistical software (Minitab Inc.; State College, PA). An empirical model was developed including a constant term and coefficients for linear and quadratic effects of each

factor as well as interaction effects between factors. Statistically insignificant ($p>0.05$) terms were sequentially removed from the model until all remaining factors were significant.

Table 2

Follow-up experiments with corn stover and corn cob pretreated under the same conditions described above used reduced β -glucosidase loadings at a constant cellulase loading of 25 FPU g⁻¹ glucan. β -glucosidase supplementation varied between 0 and 1 CBU FPU⁻¹.

Results and Discussion

Design parameters as well as glucose concentrations, expressed as a percentage of theoretical yield, for the Box-Behnken experimental design are shown in Table 3. Trends were similar for results at the different time points except that differences between some treatments were compressed at longer hydrolysis times because the yield from treatments with average rates would approach the higher rate treatments as those slowed down. Concentration data at 24 h was chosen for response surface modeling as this is more representative of hydrolysis rates and the need for high reactor productivity would discourage longer reaction times.

Table 3

The final model, developed after removing insignificant terms from the analysis and reprocessing the data, is shown in Equation (1):

$$Y = -146 + 75.5P + 2.02F - 15.2C - 7.83P^2 - 0.026F^2 + 3.35PC \quad \text{Eq (1)}$$

where:

Y = Glucose concentration (% of theoretical)

P = pH

F = Cellulase loading (FPU g⁻¹ glucan)

C = β -glucosidase loading (CBU FPU⁻¹)

The model fit the data well with an R² (adjusted) value of 0.972 and R² (prediction) of 0.923. The model also passed the fit test (p>0.05) further indicating model adequacy. All final model parameters were statistically significant (p<0.05). The ANOVA table for the model is shown in Table 4.

Table 4

Although the β -glucosidase loading was statistically significant in the model, the actual effect was much less than that of either the cellulase loading or the pH. The plot of glucose yields versus cellulase loading and pH with β -glucosidase loading held constant at the midpoint illustrates that both of those factors are significant but that the response is much more robust to changes in pH than to cellulase loading (Fig 1). Fig 2 further illustrates this point by showing the non-interaction effects of each factor. The only significant interaction in the model was that between pH and β -glucosidase loading but that only results in glucose yield differences of less than approximately 4 % across the full range of pH values (Fig 3).

Fig 1

Fig 2

Fig 3

The response with changes in β -glucosidase and cellulase loadings at a constant pH of 5.0 shows that there is very little variation in response as β -glucosidase increases within the design space (Fig 4). The model does show a significant interaction between pH and CBU loading, but results are not strikingly different at other pH values. Further, the change in response is not appreciably different at low and high cellulase loadings. The model predicts a maximum glucose concentration within the design space of 76.7 % of theoretical with the following conditions: pH of 5.3, cellulase loading of 30 FPU, and a β -glucosidase loading of 2.0 CBU FPU⁻¹. Using the optimal pH and cellulase loading conditions but a β -glucosidase loading of 0.5 CBU FPU⁻¹, however, still results in a predicted response of 73.1 %. Although the R^2 (pred) is considered strong at 0.923, it is not possible to distinguish this degree of difference between high and low β -glucosidase loadings.

Fig 4

To better understand the optimal region with the design space, we looked at how much each parameter could change while still predicting a 24-hour glucose yield of 72.9 % of theoretical which represents 95 % of the predicted optimal value (76.7 %) within the design space (Table 5). Both pH and cellulase loading had a defined region within the design space that could produce near-optimal results indicating that is important to maintain those parameters within the given range. However, it was possible to achieve 95 % of predicted optimal yields at any β -glucosidase

loading tested. So although β -glucosidase is statistically significant, the practical difference across the range of value tested was very small.

Table 5

Corn stover and corn cob were both used to test further reduction of β -glucosidase loadings at a constant loading of 25 FPU g⁻¹ glucan. The RSM model showed no significant interaction between cellulase loading and the β -glucosidase loading so no other cellulase loadings were tested. Fig 5 shows that there is a significant increase in corn stover hydrolyzate glucose concentrations between β -glucosidase supplementation of 0 and 0.2 CBU FPU⁻¹ but that there is little benefit for increasing beyond that point. Although there is a statistical difference between the stover hydrolyzates at loadings of 0.2 CBU FPU⁻¹ and those at 0.8 CBU FPU⁻¹ or 1.0 CBU FPU⁻¹, the practical difference at the 24-hour time point is likely negligible. Corn cob hydrolyzates showed a similarly large difference in glucose concentrations between no β -glucosidase supplementation and supplementation at 0.2 CBU FPU⁻¹, but no significant difference was seen when increasing any further. Zhang et al. [32] found that 24-hour glucose concentrations from soaking in aqueous ammonia pretreated corn cobs increased with β -glucosidase loadings up to 2 CBU FPU⁻¹ indicating that β -glucosidase supplementation requirements may differ based on pretreatment.

Fig 5

Residual cellobiose concentrations were also analyzed for corn stover hydrolyzates throughout the 72-hour hydrolysis (Fig 6). Cellobiose is a known strong inhibitor of cellulases and increasing concentrations would indicate higher levels of inhibition and a deficiency for further

hydrolysis to glucose [6]. Fig 6 also illustrates the large difference between no β -glucosidase supplementation and supplementation at 0.2 CBU FPU⁻¹. There are statistical differences in cellobiose concentrations as loadings increase, especially between 0.2 and 0.4 CBU FPU⁻¹, but there is no distinction between 0.4 and 1.0 CBU FPU⁻¹. Further, the differences that are seen should not have a practical impact on hydrolysis since deviations are less than 0.1 g L⁻¹. An interesting phenomenon noted in the data is that at the higher β -glucosidase loadings, residual cellobiose concentrations are higher after 72 h of hydrolysis than at 24 h. These differences again have little practical significance and could be an anomaly, but could also indicate a slight level of inhibition at the higher supplementation levels.

Fig 6

Conclusions

RSM models showed that loading β -glucosidase proportionately to *T. reesei* cellulase loading initially appears to be logical based on the lack of interaction between those factors. Performing hydrolysis at different pH levels also did not have a significant effect on the need for supplemental β -glucosidase. Although researchers may continue to use high β -glucosidase loadings of (1 to 2) CBU FPU⁻¹ based on precedent, this work shows that there is little practical benefit at an industrial scale to any loading above 0.2 CBU FPU⁻¹ for dilute acid pretreated feedstocks.

Conclusions

219 The authors wish to thank Jana Daeuber, NDSU ABEN Department, for assistance in figure
220 preparation. This work was partially supported by the USDA National Institute of Food and
221 Agriculture Hatch project ND01476.

REFERENCES

- [1] Aden A, Foust T. Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. *Cellulose*. 2009;16(4):535-45.
- [2] Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW. Technoeconomic analysis of biofuels: A wiki-based platform for lignocellulosic biorefineries. *Biomass Bioenergy*. 2010;34(12):1914-21.
- [3] Dutta A, Dowe N, Ibsen KN, Schell DJ, Aden A. An economic comparison of different fermentation configurations to convert corn stover to ethanol using *Z. mobilis* and *Saccharomyces*. *Biotechnol Progr*. 2010;26(1):64-72.
- [4] Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW. The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnol Bioeng*. 2012;109(4):1083-7.
- [5] Van Dyk JS, Pletschke BI. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy. *Biotechnol Adv*. 2012;30(6):1458-80.
- [6] Holtzapple M, Cognata M, Shu Y, Hendrickson C. Inhibition of *Trichoderma reesei* cellulase by sugars and solvents. *Biotechnol Bioeng*. 1990;36(3):275-87.
- [7] Singhania RR, Patel AK, Sukumaran RK, Larroche C, Pandey A. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresour Technol*. 2013;127(1):500-7.
- [8] Yang B, Wyman CE. Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels Bioprod Biorefin*. 2008;2(1):26-40.

245

246 [9] Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, et al. Features of promising
 247 technologies for pretreatment of lignocellulosic biomass. *Bioresour Technol.*
 248 2005;96(6):673-86.

249 [10] Eggeman T, Elander RT. Process and economic analysis of pretreatment technologies.
 250 *Bioresour Technol.* 2005;96(18):2019-25.

251 [11] Tengborg C, Galbe M, Zacchi G. Influence of Enzyme Loading and Physical Parameters on
 252 the Enzymatic Hydrolysis of Steam-Pretreated Softwood. *Biotechnol Progr.* 2001;17(1):110-
 253 7.

254 [12] Kim TH, Lee YY. Pretreatment of corn stover by soaking in aqueous ammonia. *Appl*
 255 *Biochem Biotech.* 2005;124(1-3):1119-32.

256 [13] Xu Z, Wang Q, Jiang Z, Yang X-X, Ji Y. Enzymatic hydrolysis of pretreated soybean straw.
 257 *Biomass Bioenergy.* 2007;31(2-3):162-7.

258 [14] da Costa JA, Marques JE Jr, Gonçalves LRB, Rocha MVP. Enhanced enzymatic hydrolysis
 259 and ethanol production from cashew apple bagasse pretreated with alkaline hydrogen
 260 peroxide. *Bioresour Technol.* 2015(3);179:249-59.

261 [15] Jin Y, Yang L, Jameel H, Chang H-M, Phillips R. Sodium sulfite-formaldehyde
 262 pretreatment of mixed hardwoods and its effect on enzymatic hydrolysis. *Bioresour Technol.*
 263 2013;135(5):109-15.

264 [16] Pallapolu VR, Lee YY, Garlock RJ, Balan V, Dale BE, Kim Y, et al. Effects of enzyme
 265 loading and β -glucosidase supplementation on enzymatic hydrolysis of switchgrass
 266 processed by leading pretreatment technologies. *Bioresour Technol.* 2011;102(24):11115-20.

- [17] Kim DS, Myint AA, Lee HW, Yoon J, Lee Y-W. Evaluation of hot compressed water pretreatment and enzymatic saccharification of tulip tree sawdust using severity factors. *Bioresour Technol.* 2013;144(9):460-6.
- [18] Shi J, Ebrik MA, Wyman CE. Sugar yields from dilute sulfuric acid and sulfur dioxide pretreatments and subsequent enzymatic hydrolysis of switchgrass. *Bioresour Technol.* 2011;102(19):8930-8.
- [19] Berlin A, Gilkes N, Kilburn D, Bura R, Markov A, Skomarovsky A, et al. Evaluation of novel fungal cellulase preparations for ability to hydrolyze softwood substrates – evidence for the role of accessory enzymes. *Enzyme Microb Technol.* 2005;37(2):175-84.
- [20] Bouxin FP, Jackson SD, Jarvis MC. Organosolv pretreatment of Sitka spruce wood: Conversion of hemicelluloses to ethyl glycosides. *Bioresour Technol.* 2014;151(1):441-4.
- [21] Jung YH, Kim IJ, Han J-I, Choi I-G, Kim KH. Aqueous ammonia pretreatment of oil palm empty fruit bunches for ethanol production. *Bioresour Technol.* 2011;102(20):9806-9.
- [22] Chen M, Zhao J, Xia L. Enzymatic hydrolysis of maize straw polysaccharides for the production of reducing sugars. *Carbohydr Polym.* 2008;71(3):411-5.
- [23] Geng A, Xin F, Ip J-Y. Ethanol production from horticultural waste treated by a modified organosolv method. *Bioresour Technol.* 2012;104(1):715-21.
- [24] Zhang X, Qin W, Paice MG, Saddler JN. High consistency enzymatic hydrolysis of hardwood substrates. *Bioresour Technol.* 2009;100(23):5890-7.
- [25] Ferreira S, Duarte AP, Ribeiro MHL, Queiroz JA, Domingues FC. Response surface optimization of enzymatic hydrolysis of *Cistus ladanifer* and *Cytisus striatus* for bioethanol production. *Biochem Eng J.* 2009;45(3):192-200.

- [26] Lan TQ, Lou H, Zhu JY. Enzymatic saccharification of lignocelluloses should be conducted at elevated pH 5.2–6.2. *Bioenerg Res.* 2013;6(2):476-85.
- [27] Balsan G, Astolfi V, Benazzi T, Meireles MA, Maugeri F, Di Luccio M, et al. Characterization of a commercial cellulase for hydrolysis of agroindustrial substrates. *Bioproc Biosyst Eng.* 2012;35(7):1229-37.
- [28] Samaratunga A. Efficacy and recovery of cellulases immobilized on polymer brushes grafted on silica nanoparticles [thesis]. Fargo (ND): North Dakota State University; 2014.
- [29] Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, et al. Determination of structural carbohydrates and lignin in biomass. Golden (CO): National Renewable Energy Laboratory; 2011. Technical Report NREL/TP-510-42618
- [30] Ghose TK. Measurement of cellulase activities. *Pure Appl Chem.* 1987;59(2):257-68.
- [31] Adney B, Baker J. Measurement of cellulase activities. Golden (CO): National Renewable Energy Laboratory; 2008. Technical Report NREL/TP-510-42628
- [32] Zhang M, Su R, Qi W, He Z. Enhanced enzymatic hydrolysis of lignocellulose by optimizing enzyme complexes. *Appl Biochem Biotech.* 2010;160(5):1407-14.

Fig 1 Contour plot of glucose yields after 24-hour hydrolysis of DA-pretreated corn stover while varying cellulase loading and hydrolysis pH and holding β -glucosidase supplementation constant at 1.25 CBU FPU⁻¹

Fig 2 Main effect plots for pH, cellulase loading, and β -glucosidase loading plot for mean glucose yields after 24-hour hydrolysis of DA-pretreated corn stover

Fig 3 Interaction plots for pH and β -glucosidase loading. Each line shows mean glucose yields across all cellulase loadings for the given β -glucosidase loading

Fig 4 Contour plot of glucose yields after 24-hour hydrolysis of DA-pretreated corn stover while varying β -glucosidase supplementation and cellulase loading while holding hydrolysis pH constant at 5.0

Fig 5 Glucose yields after 24-hour hydrolysis of DA-pretreated corn stover and corn cob while varying β -glucosidase supplementation and holding cellulase loading at 25 FPU g⁻¹ glucan

Fig 6 Cellobiose concentrations after 24 h, 48 h, and 72 h of hydrolysis of DA-pretreated corn stover while varying β -glucosidase supplementation and holding cellulase loading at 25 FPU g⁻¹ glucan

Table 1 Composition of original and pretreated corn stover and corn cob

Material	Solids (%)	AIL (%)	ASL (%)	Glucan (%)	Xylan (%)
Corn stover					
Original	95.5 (0.2)	18.2 (0.3)	2.0 (0.1)	41.2 (3.8)	21.3 (2.2)
DA-pretreated	21.1 (0.2)	33.2 (1.6)	1.2 (<0.1)	60.1 (0.4)	2.7 (0.3)
Corn cob					
Original	95.1 (<0.1)	15.3 (0.7)	0.4 (<0.1)	39.3 (0.4)	35.0 (0.3)
DA-pretreated	20.1 (0.7)	27.8 (1.9)	1.1 (<0.1)	72.8 (<0.1)	3.2 (0.4)

Table 2 Factor levels for Box-Behnken experimental design for hydrolysis of dilute acid pretreated corn stover.

Level	Factor		
	pH	Cellulase (FPU g ⁻¹ glucan)	β-glucosidase (CBU FPU ⁻¹)
-1	4.0	10	0.5
0	4.7	20	1.25
+1	5.4	30	2.0

Table 3 Experimental design and resulting glucose concentrations for hydrolysis of dilute acid pretreated corn stover at 24, 48, and 72 h of hydrolysis.

Design factor levels			Glucose concentrations (% of theoretical)		
pH	Cellulase (FPU)	β -glucosidase (CBU FPU ⁻¹)	24 h	48 h	72 h
4	10	1.25	67.1	72.2	82.3
5.4	10	1.25	72.9	80.6	82.5
4	30	1.25	53.6	60.4	62.3
5.4	30	1.25	68.1	73.1	82.9
4	20	0.5	66.5	69.1	69.4
5.4	20	0.5	73.3	79.6	84.1
4	20	2	64.1	73.4	77.7
5.4	20	2	55.3	61.5	69.1
4.7	10	0.5	67.1	71.8	82.0
4.7	30	0.5	60.8	67.1	66.1
4.7	10	2	57.6	59.5	60.0
4.7	30	2	76.9	82.8	86.8
4.7	20	1.25	55.9	61.4	63.6
4.7	20	1.25	44.9	47.9	52.6
4.7	20	1.25	68.0	74.9	80.6

Table 4 ANOVA for response surface model (uncoded units) of glucose concentrations after 24-h hydrolysis of DA-pretreated corn stover when varying hydrolysis pH, cellulase loading, and β -glucosidase loading.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	6	1034.54	1034.5	172.42	82.85	0.000
			4	3		
Linear	3	948.21	156.96	52.319	25.14	0.000
pH (P)	1	148.18	56.63	56.632	27.21	0.001
Cellulase (F)	1	798.60	91.98	91.980	44.20	0.000
β -glucosidase (C)	1	1.43	11.36	11.360	5.46	0.048
Square	2	73.98	73.98	73.98	17.77	0.001
P*P	1	49.82	54.63	54.632	26.25	0.001
F*F	1	24.16	24.16	24.156	11.61	0.009
Interaction	1	12.36	12.36	12.355	5.94	0.041
P*C	1	12.36	12.36	12.355	5.94	0.041
Residual Error	8	16.65	16.65	2.081		
Lack-of-Fit	6	16.02	16.02	2.670	8.51	0.109
Pure Error	2	0.63	0.63	0.314		
Total	14	1051.19				

Table 5 Minimum and maximum parameter values that can result in a predicted glucose concentration of 95% of the predicted maximum while varying the remaining parameters.

Parameter	Minimum	Maximum
pH	4.5	5.2
Cellulase loading (FPU)	23.5	30
β -glucosidase loading (CBU FPU ⁻¹)	0.5	2.0

FIGURES
FIG 1

FIG 2

FIG 3

FIG 4

FIG 5

FIG 6