POTENTIAL OF THERMOSTABLE CELLULASES IN THE BIOPROCESSING OF SWITCHGRASS TO ETHANOL

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Switchgrass (*Panicum virgatum*), a perennial grass native to North America, is a promising energy crop for bioethanol production. The aim of this study was to optimize the enzymatic saccharification of thermo-mechanically pretreated switchgrass using a thermostable cellulase from *Geobacillus* sp. in a three-level, four-variable central composite design of response surface methodology. Different combinations of solids loadings (5 to 20%), enzyme loadings (5 to 20 FPU g^{-1} DM), temperature (50 to 70 ^{o}C), and time (36 to 96 h) were investigated in a total of 30 experiments to model glucose release from switchgrass. All four factors had a significant impact on the cellulose conversion yields with a high coefficient of determination of 0.96. The use of higher solids loadings (20%) and temperatures (70 ^{o}C) during enzymatic hydrolysis proved beneficial for the significant reduction of hydrolysis times (2.67-times) and enzyme loadings (4-times), with important implications for reduced capital and operating costs of ethanol production. At 20% solids, the increase of temperature of enzymatic hydrolysis from 50 ^{o}C to 70 ^{o}C increased glucose concentrations by 34%. The attained maximum glucose concentration of 23.52 g L^{-1} translates into a glucose recovery efficiency of 46% from the theoretical yield. Following red yeast fermentation, a maximum ethanol concentration of 11 g L^{-1} was obtained, accounting for a high glucose to ethanol fermentation efficiency of 92%. The overall conversion efficiency of switchgrass to ethanol was 42%.

Keywords: Switchgrass; Bioethanol, Thermostable cellulase; Enzymatic hydrolysis; Solids loadings; Optimization; Cost efficiency; Response surface methodology

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INTRODUCTION

The global trend for production of cellulosic bioethanol from renewable resources is currently driven by three important factors: 1) increasing demand and prices of petroleum-derived fuel; 2) increasing food needs; and 3) increasing greenhouse gas emissions. Bioethanol, produced from agricultural or forest residues and dedicated energy crops, is considered to be the renewable energy with the greatest potential to replace the petroleum-derived fossil fuels of environmental concern (Cardona and Sanchez 2007; Kumar et al. 2009). In the US alone nearly 1.3 billion dry tons of biomass could be

available for large-scale bioenergy and biorefinery industries, enough to displace 30% or more of the nation's current consumption of liquid transportation (Perlack et al. 2005). In 2009 the US produced 40.69 billion liters of ethanol, and together with Brazil, accounted for 89% of the world's production.

Among the bioenergy feedstocks, switchgrass (*Panicum virgatum*), a perennial grass native to the tallgrass prairies abundantly found in North America, appears as one of the most promising plant materials for ethanol production. Based on economic and environmental impact analyses, Sanderson et al. (2006) recommended switchgrass as a biofuel feedstock that can produce five times the energy it takes to grow. Because of its excellent growth in various soil and climate conditions at yields of up to 39.5 x 10^{-4} tons sq. meter^{-1} (Lee 2006) and low fertilizer (5.6-11g nitrogen sq. meter^{-1}; Nyoka et al. 2007) and herbicide requirements for its production, a great interest has been recently developed in converting switchgrass to ethanol (Jensen et al. 2007; Keshwani and Cheng 2009). Switchgrass is relatively rich in cellulose (28.2 to 37.0%) and hemicelluloses (23.7 to 27.3%), while it contains less lignin (15.5 to 18.4%) and other non-carbohydrate components (Keshwani and Cheng 2009). Moreover, switchgrass can also serve as a carbon storage sink (Sladden et al. 1991), since the greenhouse gas (GHG) emissions from switchgrass-derived bioethanol are 94% lower than the estimated GHG emissions from gasoline production and use (Schmer et al. 2008). Therefore, as a non-food lignocellulosic biomass, switchgrass fulfills the requirements for a second-generation feedstock that can address the socio-economic challenges related to the increasing prices of fossil-based fuels, growing food needs, and increasing greenhouse gas emissions.

The enzymatic hydrolysis of the cellulosic materials has been extensively studied in the last few decades as a means to increase the yields of fermentable sugars available for ethanol production (Sun and Cheng 2002). Several factors affect the enzymatic hydrolysis of cellulose; these include chemical composition of biomass, cell wall porosity, cellulose crystallinity and accessibility, and cellulase specific activity and productivity (Alvira et al. 2010). In order to improve the yield and rate of the enzymatic hydrolysis and reduce enzyme costs, research among others has focused on enhancement of cellulase productivity and optimization of cellulose hydrolysis (Ferreira et al. 2009; Sun and Cheng 2002). *Trichoderma reesei* (Kansoh et al. 1999; Eveleigh et al. 2009) and *Aspergillus* sp. (Stewart and Parry 1981; Zambare and Christopher 2010) have been the most widely investigated sources of cellulase.

Due to improved substrate solubility and mass transfer rates, thermostable enzymes with enhanced capabilities for cellulose degradation may significantly enhance the cost efficiency of bioethanol production (Buckley and Wall 2006). Thermostable cellulases provide greater stability and reaction rates in the enzymatic hydrolysis of cellulose resulting in reduced amounts of enzyme needed for hydrolysis (Blumer-Schuette et al. 2008). This approach has the potential to further reduce the cellulase costs for ethanol production, which currently constitute more than 10% of the total costs (Bryan 2002). Furthermore, the reduced viscosity of feedstock allows the use of higher solids loadings (Kumar and Wyman 2008). Biomass slurries become viscous and difficult to mix and transport at slurry concentrations above 10%, and large volumes of water are needed to reduce viscosity and provide a flowable slurry, which limits the ethanol titers to the range 3 to 5% (Hahn-Hägerdal et al. 2006). Biomass solids loadings of at least 15%

are necessary to minimize the costs for downstream product recovery, with distinct advantages of increased productivity and ethanol concentration, lower energy requirements for heating and cooling resulting in lower operating costs, and reduced water volumes resulting in reduced disposal, treatment, and capital costs (Hodge et al. 2008).

To date, however, the use of thermophilic enzymes for cellulose hydrolysis has been scantly explored. Previously we reported on the production and characterization of thermostable cellulases from *Bacillus* and *Geobacillus* species isolated from deep gold mine environments (Rastogi et al. 2010). The thermostable enzymes were produced at 60 ^{o}C and exhibited a remarkable thermostability (e.g. 78% residual activity after incubation at 70 ^{o}C for 1 day) and broad temperature (60 to 80 ^{o}C) and pH (4 to 8) activity ranges.

Here we report on the hydrolytic potential of a *Geobacillus* sp. thermostable cellulase to degrade lignocellulosic substrates such as switchgrass. The aim of this study was to optimize the enzymatic saccharification of switchgrass at high solids and elevated temperatures, with important implications for reduced capital and operating costs of ethanol production.

This work further aims to demonstrate the advantages of using statistical methods for optimization such as response surface methodology (RSM) that can save time and costs from the reduced number of individual experiments, and that can improve the accuracy and interpretation of research findings. RSM was employed to identify the optimal conditions for hydrolysis of switchgrass by analyzing the relationships between important parameters that affect enzymatic hydrolysis such as enzyme loadings, solids loadings, temperature, and time.

EXPERIMENTAL

Materials and Chemicals

Switchgrass (variety Sunburst), harvested in summer of 2007, was obtained from a local farm in South Dakota. It was ground in a hammer mill (Speedy King, Winona Attrition Mill Co, MN) using a 4-mm sieve and then stored in sealed bins (0.68 m high with 0.47 m diameter) at room temperature (20±1°C) until use. Analytical grade 3,5-dinitrosalicylic acid (DNS) and glucose were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Extrusion Pretreatment

Switchgrass was thermo-mechanically pretreated using a single screw extruder (Brabender Plasticorder Extruder Model PL2000, Hackensack, NJ) under pre-optimized pretreatment conditions of barrel temperature (176°C), screw speed (155 rpm), moisture content (20%) and particle size (8 mm) (Karunanithy and Muthukumarappan 2011) The chemical composition of switchgrass was analyzed by Olson Biochemistry Laboratories (Brookings, SD, USA) using standard chromatography methods. The pretreated switchgrass was stored in a freezer (-18 ^{o}C) until use.

Cellulase Production

Geobacillus sp. R7 was maintained on minimal salt (MS) agar slants at 4 ˚C and as glycerol stock at –20 ˚C. The *Geobacillus* cellulase was produced with microcrystalline cellulose (MCC), untreated switchgrass, or pretreated switchgrass as a sole carbon source under aerobic liquid fermentative conditions (60 ^{o}C, pH 7.0, 150 rpm) for 7 days. The MS medium contained MCC or switchgrass (untreated or pretreated), 0.5 g L^{-1}; nitrilotriacetic acid, 0.1 g L^{-1}; 0.03% $FeCl_3$ solution, 1 mL L^{-1}; $CaCl_2.2H_2O$, 0.05 g L^{-1}; $MgSO_4.7H_2O$, 0.1 g L^{-1}; NaCl, 0.01 g L^{-1}; KCl, 0.01 g L^{-1}; NH_4Cl, 0.3 g L^{-1}; 85% H_3PO_4, 1.8 g L^{-1}; methionine, 0.005 g L^{-1}; yeast extract, 0.05 g L^{-1}; casamino acids, 0.01 g L^{-1}, and Nitsch's trace element solution, 1 mL L^{-1} (Rastogi et al. 2009). The fermentation broth was centrifuged at 5,000 rpm for 10 min. The supernatant was concentrated by ammonium sulphate precipitation (80% saturation) and dialyzed at 4 ^{o}C against the 100 mM phosphate buffer (pH 6). The dialyzed enzyme was used for enzymatic hydrolysis of pretreated switchgrass.

Cellulase Assays

The cellulase activity was calculated using carboxymethyl cellulose (CMC) as substrate. A suitably diluted enzyme (0.1 mL) was incubated with 0.5 mL of CMC solution (1%, wv^{-1}) in acetate buffer (0.1 M, pH 6) at 70 ^{o}C for 20 min. The reaction was stopped by addition of 1.0 mL of DNS. The mixture was boiled for 10 min and allowed to cool to room temperature.

The reducing sugars (glucose) were determined by measuring the absorbance of the enzymatic hydrolysates at 540 nm (Miller 1959). One unit (U) of cellulase activity (CMCase) was defined as the amount of enzyme that released 1 μmol of glucose per minute under the assay conditions.

Likewise, the filter paper activity was measured with 0.1 g of Whatman filter paper No.1 as substrate (Ghose 1987). One filter paper unit (FPU) was defined as the amount of enzyme that released 1 μmol of glucose per minute under the assay conditions.

Enzymatic Hydrolysis

The values of test variables at different enzyme loadings (5-20 FPU g^{-1}DM), solids loadings (5 to 20%), temperature (50 to 70 ^{o}C), and time (36 to 96 h) coded from minus 1 to 1, and their interactions according to CCD are shown in Table 2. For enzymatic hydrolysis, different solids loadings of extrusion pretreated switchgrass (5 to 20%) were weighed in 25 mL capacity screw-cap bottles. Before adding the enzyme, the bottles containing switchgrass and 100 mM phosphate buffer (pH 6) were sterilized in an autoclave (121°C, 20 min). Aliquots of filter-sterilized enzyme was aseptically added to the bottles to obtain final enzyme loadings from 5 to 20 FPU g^{-1}DM, and the whole mixture of 10 mL was then incubated at three different temperatures (50, 60, and 70 ^{o}C) under shaking condition (150 rpm). Samples were withdrawn from the bottles after 36, 66, and 96 h of enzymatic hydrolysis and centrifuged at 5,000 rpm for 10 min. The clear supernatants (enzymatic hydrolysates) were analyzed for glucose using 2700 Biochemistry Analyzer (YSI Life Sciences, Yellow Spring, OH) as per the manufacturer's instructions.

Ethanol Fermentation

Red yeast, a commercial yeast product in a granular form (Fermentis, Boubaixbr, France), was used for ethanol fermentation (Murthy et al. 2009). The strain has a high ethanol tolerance (up to 18%, vv^{-1}) at higher temperatures (up to 40 °C). Due to its higher cell viability, it is suitable for use in very high gravity processes (up to 36% solids) and simultaneous saccharification and fermentation processes (Vezzu et al. 2009). It was grown in yeast extract glucose (YG) medium containing 2 g L^{-1} yeast extract, 2 g L^{-1} glucose, and 5 mL of tetracycline solution. Inoculum was developed by incubating the yeast culture in an Erlenmeyer flask containing 100 mL of YG medium at 30 °C under shaking conditions (150 rpm) for 18 h. The enzymatic hydrolysates obtained after incubation times of 36, 66, and 96 h were supplemented with 1 mL of sterile yeast extract (2%, wv^{-1}), inoculated with 0.2 mL of Fermentis red yeast and incubated at 30 °C and 150 rpm. Samples were withdrawn after 72 h of fermentation and centrifuged at 5,000 rpm for 10 min. The clear supernatants were analyzed for their ethanol content using YSI 2700 Biochemistry Analyzer.

Experimental Design

To optimize the sugar recovery from pretreated switchgrass, a four-variable central composite design (CCD) was used to study the response pattern and determine the optimum combination of enzyme loadings (5 to 20 FPU g^{-1} DM), solids loadings (5 to 20%), temperature (50 to 70 °C), and time (36 to 96 h), which were coded from -1 to 1 levels (Table 2). The enzyme used in the experimental design studies was produced on extrusion pretreated switchgrass (Fig. 1). The CCD combines the vertices of the hypercube whose coordinates are given by a 2^n factorial design with star points that provide estimation of curvature for the nonlinear response surface (Garcia et al. 1999). The experimental design was developed using Design Expert 8 (Statease, Minneapolis, MN, USA). A total of 30 experiments (16 factorial, 8 axial, and 6 center points) were randomized to maximize the effects of unexplained variability in the observed responses, which were due to extraneous factors. The independent variables were coded according to Eq. 1,

$$x_i = (X_i - X_0)/\,\Delta X_i \qquad (1)$$

where x_i and X_i are the dimensionless and actual values of the independent variable, respectively; X_0 is the actual value of the independent variable at the center point; and ΔX_i is the step change of X_i corresponding to unit variation of the dimensionless value. The selected variables (enzyme loadings, solids loadings, temperatures, and incubation time) were studied at three levels: −1, 0, and 1 (Table 2).

Statistical Analysis

The second order polynomials were calculated with Design-Expert Version 8 software to estimate the response of the dependent variables (Eq. 2).

$$Y_1 = b_0 + \sum_{i=1}^{4} b_i X_i + \sum_{i=1}^{4} b_{ii} X_{i^2} + \sum_{i=1}^{3} \sum_{j=i+1}^{4} b_{ij} X_i X_j + \epsilon \qquad (2)$$

where Y_i is the predicted response for glucose concentration in g L^{-1}; b_0 is the intercept; b_i is the coefficient for linear direct effect; b_i^2 is the coefficient for quadratic effect; b_{ij} is the coefficient for interaction effect (a positive or negative significant value implies possible interaction between the medium constituents); ε is the random error; and X_i, X_i^2, and X_{ij} are the independent variables studied.

The quality of fit for the second order equation was expressed by the coefficient of determination (R^2), and its statistical significance was determined by the F-test. The effect of each independent variable and their interaction effects were determined. The number of parameters that were chosen to be included for each model were determined based on the significance (α = 0.05) of each model parameter using the F-test. To maximize the sugar recovery from switchgrass, numerical optimization was used for determination of the optimal levels of the four variables. An experimental run of enzymatic hydrolysis followed by ethanol fermentation was conducted under optimal conditions (17.18 FPU g^{-1}, 20% solids loading, 70°C, 96 h). All experiments were performed in triplicate, and standard deviations were calculated from the mean of triplicate analyses.

RESULTS AND DISCUSSION

Cellulase Production

As evident from Table 1, the extrusion pretreatment did not alter the carbohydrate composition of switchgrass (Table 1). However, the thermo-mechanical extrusion of switchgrass appeared to have produced a positive effect on the cellulase activity (Fig. 1). For instance, pretreatment of switchgrass resulted in a 2.76-fold increase in the cellulase activity (from 31.30 U L^{-1}, on switchgrass, to 86.70 U L^{-1}, on pretreated switchgrass). This represented a 1.46-fold increase over the maximum cellulase (CMCase) activity of 59 U L^{-1} produced when R7 was grown on MCC. Switchgrass was also used for solid-state production of xylanase and cellulase by *Acidothermus cellulolyticus* (Rezaei et al. 2011). Biomass pretreatment, depending on the type and severity, is known to impact cellulose crystallinity, biomass porosity, and fractionation (Sun and Cheng 2002). Thermo-mechanical extrusion was previously shown to improve the sugar recovery yields (Karunanithy and Muthukumarappan 2010) due to partial removal of lignin and inhibitory compounds (Kumar et al. 2009). We also reported a 32.1% increase in the cellulase production in *Geobacillus* sp. R13 on extrusion pretreated corn stover over untreated corn stover (Rastogi et al. 2010).

Table 1. Carbohydrate Composition of Untreated and Extrusion Pretreated Switchgrass

Parameters	Untreated switchgrass (%)	Extrusion pretreated switchgrass (%)
Glucose	25.5 ± 5.8	25.2 ± 3.5
Xylose	17.4 ± 2.1	17.6 ± 2.0
Arabinose	4.9 ± 1.2	4.8 ± 1.3

± indicating a standard deviation of triplicate analysis

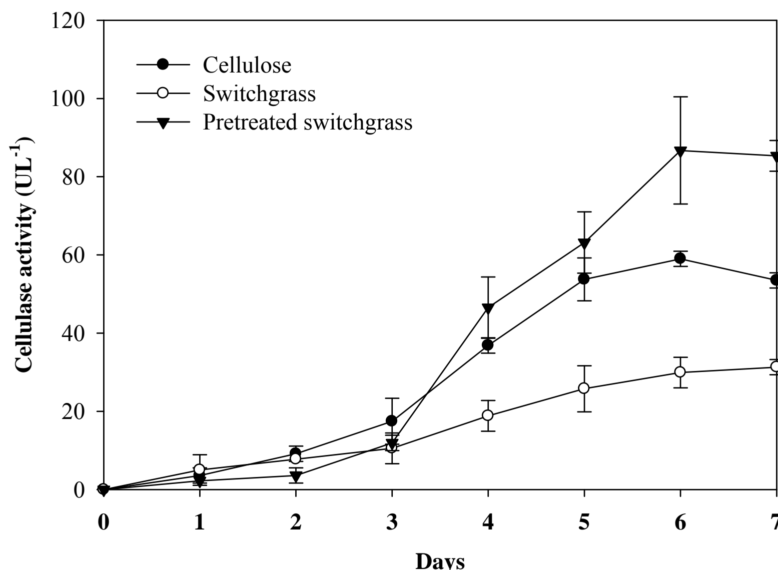


Fig. 1. Cellulase production by *Geobacillus* sp. R7 on lignocellulosic substrates (0.5 g substrate L^{-1}, 60 °C, pH 7, 150 rpm). Error bars indicate standard deviation between replicates (*n*=3).

Optimization of Enzymatic Hydrolysis

The statistical combination of the test variables along with the measured response values, which were expressed as glucose released, are summarized in Table 2. The application of the RSM yielded a regression equation, which was an empirical relationship between the reducing sugar production and the test variables in coded units. The overall second-order polynomial equation for the enzymatic saccharification was as follows:

$$\text{Glucose concentration (g L}^{-1}\text{)} = 6.92 + 1.75X_1 + 4.65X_2 + 1.63X_3 + 1.64D + 0.95X_{12} + 0.21X_{13} + 0.24X_{14} + 0.20X_{23} + 1.08X_{24} + 0.47X_{34} - 2.21X_1^2 - 1.25X_2^2 + 4.77X_3^2 + 1.39X_4^2 \quad (3)$$

In Eq. 3, X_1 is the enzyme loadings (FPU g^{-1} DM), X_2 is the solids loadings (%), X_3 is the temperature (°C), and X_4 is the time (h). The statistical significance of the model equation was checked with the *F*-test, and the analysis of variance (ANOVA) for the response surface quadratic model is shown in Table 3. The model *F*-value of 26.83 and the values of probability (P) > F (<0.0001) showed that the model terms were significant at the 95% confidence level (Box et al. 1978). The coefficient of determination (R^2), which was calculated for the glucose production, indicated that the statistical model explained 96.16% of the variability in the response. Linear terms, two quadratic terms, and two interaction terms were significant. The coefficients of the response surface model are also presented in Eq. 3. A *P*-value greater than 0.05 indicated that a term was not significant. In this case, X_1, X_2, X_3, X_4, X_{12}, X_{24}, X_1^2 and X_3^2 were the significant model terms. The linear models for each response generated response surfaces. The response for the highest glucose concentration was determined. A model is stronger and the predicted response is better as the R^2 value becomes closer to 1.0 (Gunawan et al. 2005). The adequate

precision measured the signal-to-noise ratio. Ratios greater than 4 indicated adequate model discrimination. The adequate precision of the developed model was 22.51 (Table 3), indicating that the model could be used to navigate the design space.

Table 2. Experimental Design Showing Coded and Actual Values of Variables, Observed and Predicted Responses

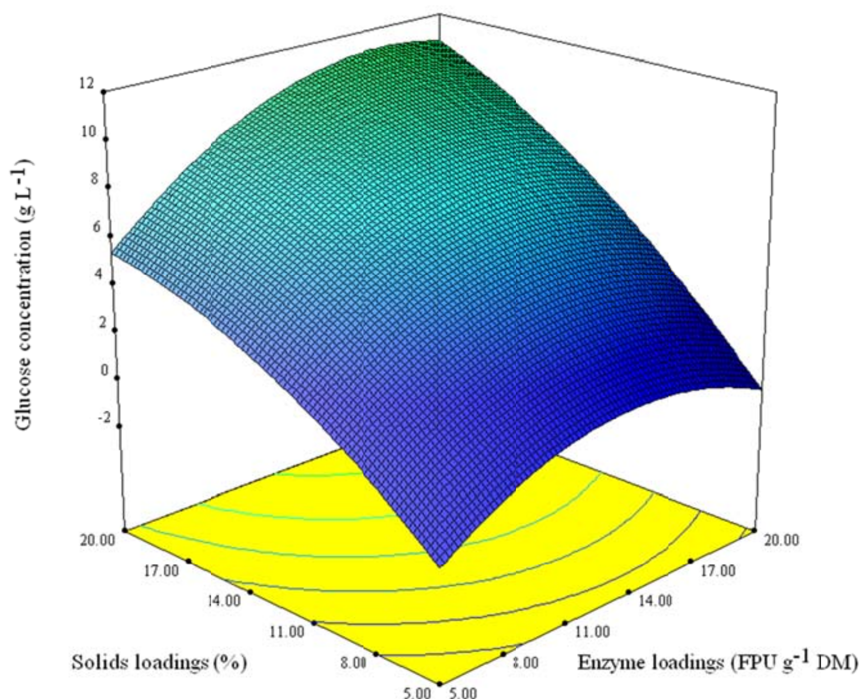
Run	Enzyme loadings	Solids loadings	Temperature	Time	Glucose concentration (g L^{-1}) Observed	Predicted	Residuals	Ethanol concentration (g L^{-1})
1	-1 (5)	-1 (5)	-1 (50)	-1 (36)	2.23	3.08	-0.85	0.99
2	1 (20)	-1 (5)	-1 (50)	-1 (36)	3.45	3.79	-0.34	1.55
3	-1 (5)	1 (20)	-1 (50)	-1 (36)	8.73	7.95	0.77	3.85
4	1 (20)	1 (20)	-1 (50)	-1 (36)	13.10	12.44	0.65	5.98
5	-1 (5)	-1 (5)	1 (70)	-1 (36)	4.56	4.61	-0.05	2.06
6	1 (20)	-1 (5)	1 (70)	-1 (36)	6.14	6.14	-0.00	2.83
7	-1 (5)	1 (20)	1 (70)	-1 (36)	10.34	10.26	0.07	4.80
8	1 (20)	1 (20)	1 (70)	-1 (36)	15.59	15.57	0.01	7.00
9	-1 (5)	-1 (5)	-1 (50)	1 (96)	2.89	2.78	0.10	1.34
10	1 (20)	-1 (5)	-1 (50)	1 (96)	4.60	4.45	0.14	2.12
11	-1 (5)	1 (20)	-1 (50)	1 (96)	12.20	11.97	0.22	5.66
12	1 (20)	1 (20)	-1 (50)	1 (96)	17.60	17.42	0.17	8.10
13	-1 (5)	-1 (5)	1 (70)	1 (96)	5.75	6.18	-0.43	2.58
14	1 (20)	-1 (5)	1 (70)	1 (96)	8.03	8.68	-0.65	3.59
15	-1 (5)	1 (20)	1 (70)	1 (96)	16.63	16.16	0.46	7.77
16	1 (20)	1 (20)	1 (70)	1 (96)	23.52	22.43	1.08	11.00
17	-1 (5)	0 (12.5)	0 (60)	0 (66)	2.66	2.96	-0.30	1.21
18	1 (20)	0 (12.5)	0 (60)	0 (66)	5.39	6.45	-1.06	2.49
19	0 (12.5)	-1 (5)	0 (60)	0 (66)	3.12	1.01	2.10	1.42
20	0 (12.5)	1 (20)	0 (60)	0 (66)	6.85	10.32	-3.47	3.01
21	0 (12.5)	0 (12.5)	-1 (50)	0 (66)	9.18	10.05	-0.87	4.25
22	0 (12.5)	0 (12.5)	1 (70)	0 (66)	12.83	13.32	-0.49	6.01
23	0 (12.5)	0 (12.5)	0 (60)	-1 (36)	6.41	6.67	-0.26	2.83
24	0 (12.5)	0 (12.5)	0 (60)	1 (96)	8.85	9.95	-1.10	3.96
25	0 (12.5)	0 (12.5)	0 (60)	0 (66)	7.55	6.92	0.62	3.33
26	0 (12.5)	0 (12.5)	0 (60)	0 (66)	7.49	6.92	0.56	3.49
27	0 (12.5)	0 (12.5)	0 (60)	0 (66)	7.30	6.92	0.37	3.41
28	0 (12.5)	0 (12.5)	0 (60)	0 (66)	7.77	6.92	0.84	3.63
29	0 (12.5)	0 (12.5)	0 (60)	0 (66)	7.96	6.92	1.03	3.56
30	0 (12.5)	0 (12.5)	0 (60)	0 (66)	7.58	6.92	0.65	3.09

The adjusted R^2 value corrected the R^2 value for the sample size and the number of terms (Jabasingh and Nachiyar 2010). The high value for the adjusted determination coefficient of 0.92 indicated a high significance of the model. The data analysis showed that solids loading as an independent variable presented the strongest and most positive effect on all responses studied, and for this reason, it was held at the highest level of 20% in order to study the combination of the other variables.

Table 3. Analysis of Variance (ANOVA) for Enzymatic Hydrolysis of Extrusion Pretreated Switchgrass

Source	Sum of Squares	DF	Mean Square	*F* Value	*p*-Value (Prob > *F*)
Model	680.39	14	48.59	26.83	< 0.0001*
X_1	54.88	1	54.88	30.30	< 0.0001*
X_2	390.04	1	390.04	215.38	< 0.0001*
X_3	48.05	1	48.05	26.53	0.0001*
X_4	48.41	1	48.41	26.73	0.0001*
X_{12}	14.28	1	14.28	7.89	0.0132*
X_{13}	0.68	1	0.68	0.37	0.5490
X_{14}	0.93	1	0.93	0.51	0.4843
X_{23}	0.62	1	0.61	0.34	0.5683
X_{24}	18.71	1	18.70	10.32	0.0058*
X_{34}	3.53	1	3.53	1.95	0.1827
X_1^2	12.66	1	12.66	6.99	0.0184*
X_2^2	4.05	1	4.05	2.23	0.1554
X_3^2	0.61	1	58.93	32.54	< 0.0001*
X_4^2	18.70	1	5.03	2.78	0.1161
Residual	3.53	1	1.81	-	-
Core total	707.56	29	-	-	-

X_1, enzyme loadings, X_2, solid loadings, X_3, temperature, X_4, time; DF, degree of freedom; Coefficients of determination (R^2), 0.96; Correlation coefficient (adjusted R^2), 0.92; Predicted coefficient R^2, 0.83; Adeq. Precision, 22.51; *Significant at 95% confidance level



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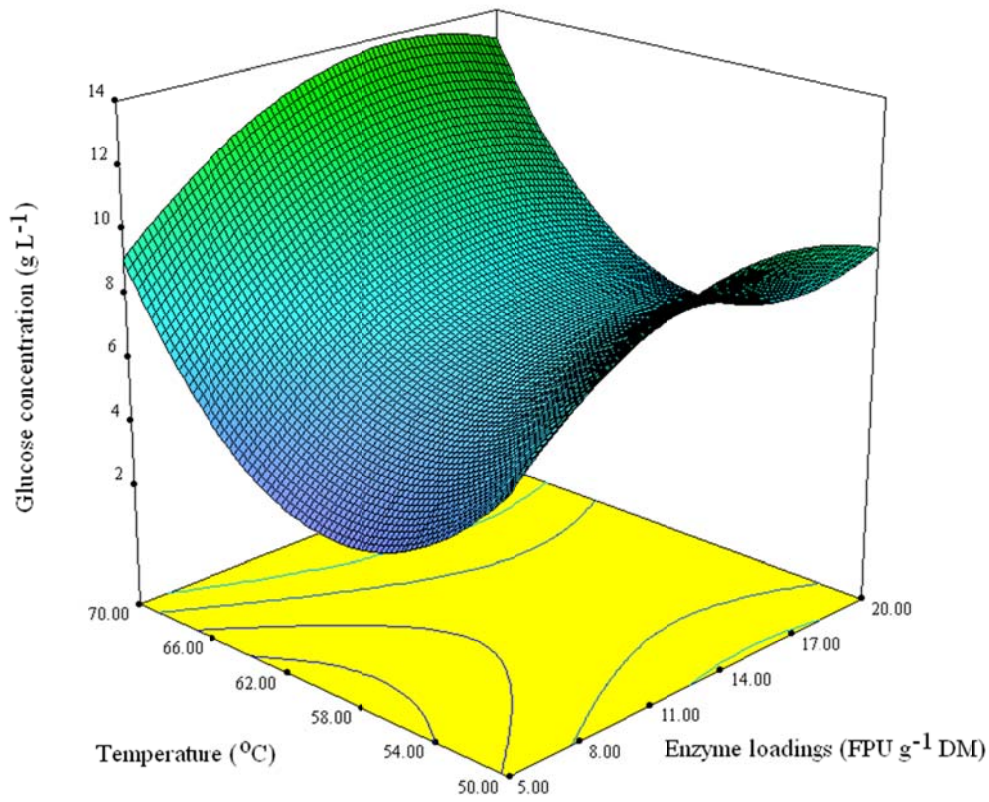

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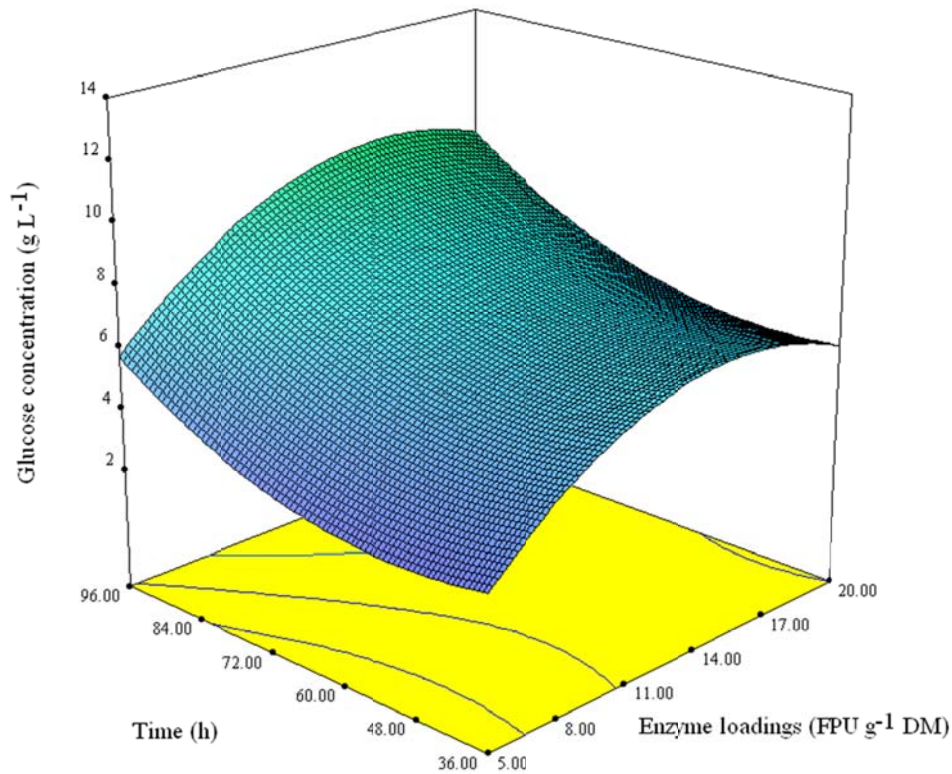

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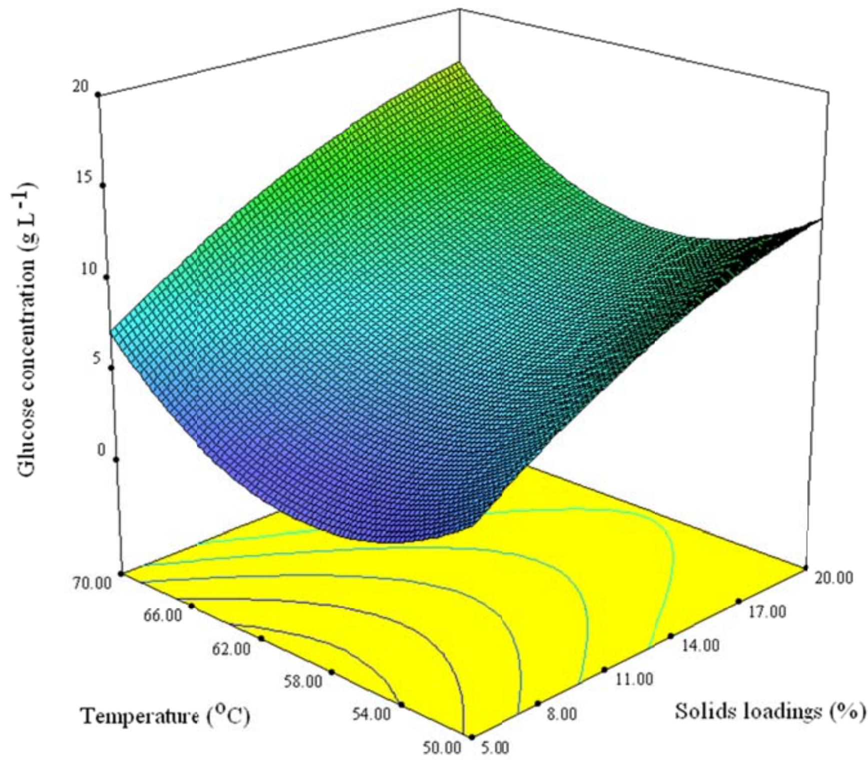

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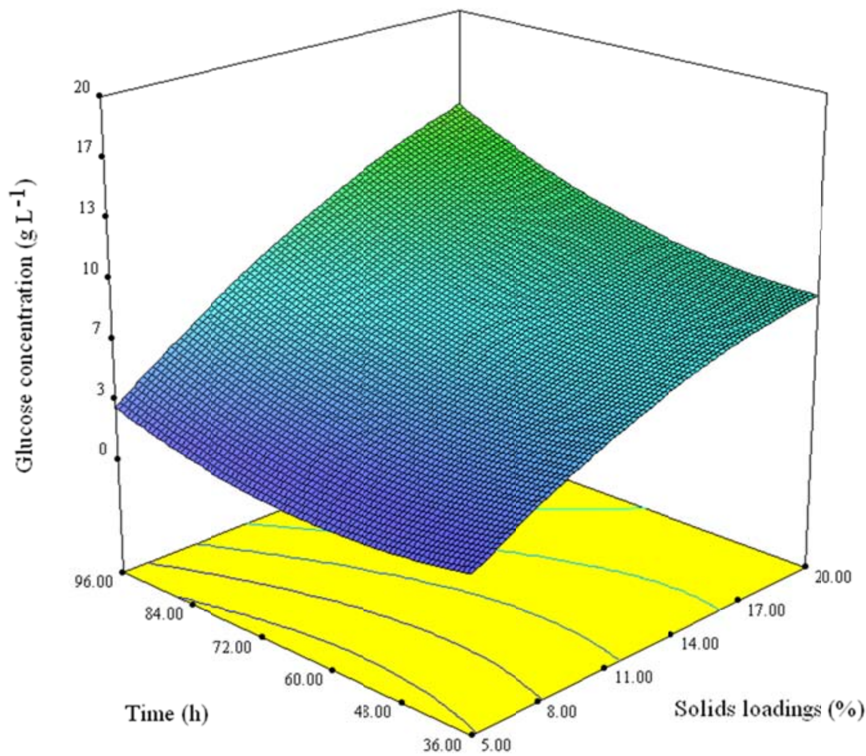

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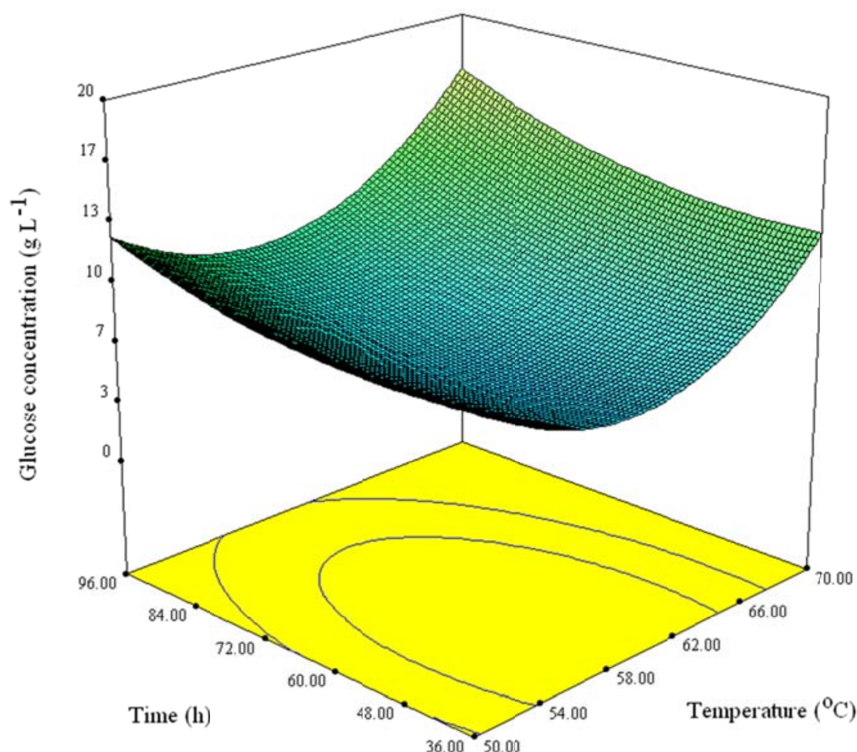

(f)

Fig. 2. Response surfaces generated by equation models for the effects of enzyme and solids loadings (a), enzyme loadings and temperature (b), enzyme loadings and time (c), solids loadings and temperature (d), solids loadings and time (e) temperature and time (f) and their mutual effects on enzymatic hydrolysis of pretreated switchgrass

Advantages of Thermostable Cellulases

As evident from Fig. 2a, the glucose concentration of 5.44 g L^{-1} obtained at lower enzyme loadings (5 FPU g^{-1} DM), more than doubled (11.13 g L^{-1}) by increasing the enzyme loadings by a factor of 4 while maintaining the solids loadings at 20%. Similar glucose concentrations (9.12-9.38 g L^{-1}) were produced at lower enzyme loadings (5 FPU g^{-1} DM) and higher temperature (70 ^{o}C), on one hand, and at higher enzyme loadings (20 FPU g^{-1} DM) and lower temperature (50°C), on the other (Fig. 2b). This clearly demonstrates the advantages of using thermostable cellulases at elevated temperatures in lowering the enzyme costs during hydrolysis of lignocellulose to fermentable sugars (Table 4). The lower glucose concentration of 7.11 g L^{-1} at 60 ^{o}C (Fig. 2b) when compared to 50 ^{o}C (10.30 g L^{-1}) and 70 ^{o}C (12.93 g L^{-1}) may be due to the presence in the crude enzyme of two cellulase isozymes with temperature optima of 50 ^{o}C and 70 ^{o}C (Kocher et al. 2008). Fig. 2c revealed that a comparable glucose recovery of 5.76 to 5.99 g L^{-1} was achieved as a trade-off between the use of lower enzyme loadings (5 FPU g^{-1} DM) at longer hydrolysis times (96 h) vs. higher enzyme loadings (20 FPU g^{-1} DM) at shorter hydrolysis times (36 h).

As per Fig. 2d, at solids loadings of 20%, the increase in hydrolysis temperature from 50 ^{o}C to 70 ^{o}C resulted in the additional release of 3.67 g glucose L^{-1} from

pretreated switchgrass. This represents a 28% increase (Table 4) in the glucose concentration from 13.24 to 16.91 g L^{-1}.

Three times more glucose was released from pretreated switchgrass (9 g L^{-1}) when the enzymatic hydrolysis was carried out at higher solids loadings (20%) for shorter hydrolysis times (36 h) when compared to the glucose recovery attained at 5% solids loadings for 96 h (Fig. 2e). The use of higher temperatures to achieve the same glucose concentration of 12.60 g L^{-1} resulted in a significant reduction of the hydrolysis time from 96 h, at 50 ^{o}C, to 36 h, at 70 ^{o}C (Fig. 2f, Table 4). This is an important observation, as long residence times have been reported to add significantly to the capital and operating costs and, consequently, to the overall biomass to ethanol conversion process (Krishna and Chowdary 2000).

As a result of the RSM numerical optimization, the highest glucose concentration of 23.62 g L^{-1} was achieved with an enzyme loadings of 17.18 FPU g^{-1} DM, solids loadings of 20%, and a temperature of 70 ^{o}C (96 h). To the best of our knowledge, this is the first report on enzymatic hydrolysis of switchgrass with thermostable cellulases at 70 ^{o}C.

Table 4. Advantages of Enzymatic Hydrolysis of Pretreated Switchgrass at 70 ^{o}C vs 50 ^{o}C

Parameter	Advantages
Enzyme loadings	4-times lower enzyme loadings required (5 FPU g^{-1} DM vs 20 FPU g^{-1} DM) to produce a similar glucose concentration (Fig. 2b)
Solids loadings	Higher glucose concentrations of 28% and 61% obtained at the same solids loadings of 20% and 5%, respectively (Fig. 2d)
Hydrolysis time	2.67-times shorter hydrolysis times required (36 h vs 96 h) to produce a similar glucose concentration (Fig. 2f)

Ethanol Fermentation

The hydrolysates obtained from pretreated switchgrass under different enzymatic hydrolysis conditions were fermented to ethanol using Fermentis red yeast (data not shown). A maximum ethanol concentration of 11.00 g L^{-1} was obtained from an initial glucose concentration of 23.52 g L^{-1} (experimental run no. 16, Table 2). This corresponds to an ethanol yield of 0.47 g ethanol g^{-1} glucose accounting for a high fermentation efficiency of 92%.

Model Validation

To validate the model, an experimental run under the optimal conditions for enzymatic hydrolysis of pretreated switchgrass was carried out. The validation experiment produced a glucose concentration of 23.62 g L^{-1} (corresponding to a 46% glucose recovery efficiency from theoretical yield), which is in good agreement with the predicted value of 22.51 g L^{-1}. Based on a 92% fermentation efficiency, the overall conversion efficiency of switchgrass to ethanol attained was 42%.

CONCLUSIONS

1. In this work, modeling of enzymatic hydrolysis of extrusion-pretreated switchgrass with thermostable cellulases of *Geobacillus* sp. was successfully performed using a central composite design (CCD) and response surface methodology (RSM).
2. The effects of solids loadings, enzyme loadings, hydrolysis temperature, and hydrolysis time were investigated as independent variables in a total of 30 experiments. All four factors were found to have a significant impact on the cellulose conversion yields.
3. The ANOVA for the enzymatic hydrolysis showed a high coefficient of determination (R^2) of 0.96 with a relationship of all four parameters (enzyme loadings of 17.18 FPU g^{-1} DM, solids loadings of 20%, temperature of 70 ^oC, and hydrolysis time of 96 h) for a maximum glucose concentration from pretreated switchgrass of 23.52 g L^{-1} (23.62 g L^{-1} – after experimental validation).
4. It was demonstrated that the use of higher solids loadings (20%) and temperatures (70 ^oC) during enzymatic hydrolysis were beneficial for the increase in the glucose recovery rates and the significant reduction of hydrolysis times (2.67-times) and enzyme loadings (4-times). At 20% solids, enzymatic hydrolysis of pretreated switchgrass at 70 ^oC increased the glucose concentration by up to 34% over that obtained at 50 ^oC.
5. Following red yeast fermentation, a maximum ethanol concentration of 11.00 g L^{-1} (0.47 g ethanol g^{-1} glucose) was obtained, indicating a high glucose to ethanol fermentation efficiency of 92%.
6. Under optimum enzymatic hydrolysis conditions, a glucose recovery efficiency of 46% from the theoretical maximum was achieved. Hence, the overall conversion efficiency from switchgrass to ethanol was 42%.
7. High solids bioprocessing of switchgrass at elevated temperatures to ethanol could bring about significant savings of capital and operating costs, as it reduces the needed enzyme loadings and hydrolysis time, size of reaction vessels, water usage, and waste water treatment costs.
8. The use of RSM for optimization of process parameters saves both time and costs, maximizing the amount of information that can be obtained while limiting the number of individual experiments. Furthermore, the RSM predicts the interaction between the independent variables, which results in improved accuracy and precision of the research data and facilitates their interpretation.

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