Optimization of Enzymatic Hydrolysis of Dilute Acid Pretreated Sugar Beet Pulp Using Response Surface Design

E. Donkoh[1], J. Degenstein[1], M. Tucker[2], Y. Ji[1*]

[1]Department of Chemical Engineering, University of North Dakota, Grand Forks, ND 58203 and
[2]National Renewable Energy Laboratory, Golden, CO 80401
Corresponding author: Yun Ji (yun.ji@engr.und.edu)

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ABSTRACT

In this study, response surface methodology (RSM) was used to optimize the enzymatic digestibility of sugar beet pulp (SBP). The reaction temperature, enzyme concentration, and SBP loading were studied using a five-level central composite design. Minitab 15 software was used to perform statistical analysis of the data. The result showed that reaction temperature and enzyme concentration have significant effect on the response. The effect of SBP percent solids was found to be insignificant. A quadratic model was developed. A maximum yield of 85% was predicted with the model. Model validation experiments showed good agreement between the actual obtained yields and the predicted yields. Therefore, the model could be used to optimize the enzymatic hydrolysis of SBP process.

Additional key words: sugar beet pulp, lignocellulosic, biomass, ethanol, pretreatment, enzymatic hydrolysis, fermentable sugar, response surface

INTRODUCTION

The recent unrest in Northern Africa and the Middle East has contributed to the increase in prices of petroleum. Alternative fuels can be used to reduce our dependence on foreign oil. Bioethanol could be a substitute for fossil fuel. Presently, most of the bioethanol produced in the United States is derived from corn. However, corn is a valuable source of food for humans and animals. Nevertheless, dedicating all the United States corn production to bioethanol would meet only 15% of annual gasoline consumption (Hill et al., 2006). The Energy Independence and Security Act of 2007 requires 36 billion gallons of renewable fuels by 2022 (Sissine, 2007). To meet this requirement, there is a need to investigate alternative feedstocks for bioethanol production.

Ethanol from lignocellulosic material is advantageous because this material is abundantly available with minimum cost. Sugar beet pulp is an attractive feedstock for ethanol production, because it is a coproduct from the table sugar industry. Sugar beet is cultivated throughout the world in temperate climates. However, in the U.S., sugar beet farming is concentrated in the Northern plains, North Dakota, South Dakota, Minnesota, Montana, Intermountain West, and Rocky Mountain area. Sugar beet pulp consists of 20–24% cellulose, 25–36% hemicellulose, 20–25% pectin or uronic acids, 1–2% lignin, and 7–8% protein, all expressed as a percentage of dry weight of total solids (Foster et al., 2001). The use of SBP addresses some of the logistical constraints most biomass feedstocks face. These constraints include: feedstock harvest, feedstock prices, transportation, and storage. Beet harvesting equipment and transportation methods are well established, and delivery of the product to the sugar processing plant is already in place. After sucrose extraction, the remaining pulp often is dried, pelletized, and sold to farmers as animal feedstock. Using SBP as a feedstock for bioethanol production would be more profitable than processing for animal feed because of the low price and limited market for animal feed.

A typical biomass to ethanol process consists of pretreatment, enzymatic hydrolysis, fermentation, and a product recovery unit operation. Because of the recalcitrant nature of biomass, each of these stages is complex and involves different factors. The pretreatment and enzymatic hydrolysis steps are the most expensive capital cost components in ligncellulosic ethanol production (Wooley et. al, 1999). Successful commercialization of the biomass to ethanol process depends on reducing the cost of the aforementioned processes. The enzymatic hydrolysis process is slow and currently exhibits low yield. To increase the yield, the enzyme loading has to be increased to high levels but enzymes are expensive. Wooley et al. (1999) also reported that the enzyme cost is second highest operating cost after biomass feedstock cost. Enzymatic hydrolysis is affected by different factors

including, degree of polymerization, degree of crystallinity, biomass structure, and available surface area (Qi et. al, 2009). The type of pretreatments used also contributes to the yield of enzymatic hydrolysis by introducing degradation products that can inhibit enzyme performance. Enzymatic hydrolysis processes are inhibited by high substrate and glucose concentrations. As a result, numerous optimization studies have been conducted and presented in the literature (Esteghlalian et. al, 1997; Chamy et. al, 1994; Ferreira et. al, 2009; Qi et. al, 2009; Schell et. al, 1999).

Little work has been reported in the literature optimizing enzymatic hydrolysis of SBP with response surface methodology. The aim of the present research was to develop a mathematical model to predict and optimize glucose yield in the enzymatic hydrolysis of sugar beet pulp. A central composite design with the aid of Minitab statistical software was used to determine the optimal levels of effective factors that would produce maximum glucose yield.

MATERIALS AND METHODS

Sugar beet pulp used in this study was obtained from American Crystal Sugar Company in East Grand Fork, MN. The SBP received contained 78% moisture. SBP was stored at -20°C when not in use. Prior to dilute sulfuric acid pretreatment, the SBP was washed to remove excess sugars. The cellulase enzyme (GC220) used in this work was obtained from Genencor in NY with protein content of 212 mg protein/mL. Cellulase was stored at 4°C when not in use.

Pretreatment of SBP

Before enzymatic hydrolysis, SBP was pretreated with dilute sulfuric acid to increase enzyme accessibility. A 300 ml Hastelloy C-276 batch reactor from Autoclave Engineers (Autoclave Engineers, Erie, Pennsylvania) was used to control corrosion and limit the effect of chromium and molybdenum metal ion leaching on pretreatment yield. SBP pretreatment was conducted at 150°C with 1.1% (w/w) sulfuric acid and 10% (w/w) solid loading for a total residence time of 12 min. After pretreatment, the slurry was washed with 3 volumes of 500 ml of deionized water and filtered under vacuum to remove the dissolved sugars. The pH of the slurry residue (with 70-80% moisture) was adjusted with sodium hydroxide to 4.8. The glucose and xylose in slurry and rinsate were measured to determine the remaining glucose. The solid compositions were determined following the guidelines of NREL laboratory analytical procedure (Sluiter et. al, 2008).

Enzymatic Hydrolysis

Hydrolysis experiments were performed in 125 ml Erlenmeyer flasks with a total working volume of 100 ml. SBP was weighed and added to 0.05 M citric sodium buffer (pH 4.8) solution (90% working

volume). The required enzyme was measured and added to buffer solution in separate flasks (10% working volume). Both SBP and enzyme solutions were placed in a rotary shaker at 180 rpm for 30 min. To initiate the reaction, the enzyme solutions were added to SBP solution. Sodium azide (0.04% [w/v]) was added to prevent microbial contamination. Hydrolysis proceeded for 72 h. Samples taken for analysis were placed in boiling water bath for 10 min to deactivate the enzymes and then stored at -20°C.

Analytical Methods

The reducing sugars were quantified by high performance liquid chromatography (HPLC). The hydrolysis samples were filtered through micro filter (0.2 μm). Glucose and cellobiose content from the hydrolysis experiments were determined using a polymer column (Transgenomic CHO-782Pb, Omaha, NE) at 85°C. The percent hydrolysis yield was obtained using the relation developed by NREL (Dowe and McMillan, 2008)

$$\%\ Yield = \frac{(Glucose) + 1.053 \cdot (Cellobiose)}{1.111 \cdot f \cdot (Biomass)} \cdot 100\%$$

The multiplication factor 1.053, converts cellobiose to equivalent glucose and *f* is the cellulose fraction in dry biomass (g/g) (Dowe and McMillan, 2008). Glucose is the residual glucose concentration obtained with HPLC in g/L, Cellobiose is the residual cellobiose concentration obtained with HPLC in g/L, and Biomass is the dry biomass concentration at the beginning of the enzymatic hydrolysis.

Experimental Design

A response surface methodology (RSM) is used to describe in detail the relationship between the factors and a response (Lawson and Erjavec, 2001). The steps generally involved in successful application of RSM are design and collection of experimental data, which allows fitting a general quadratic equation for smoothing and prediction, performing regression analysis to select the best equation for description of the data, and examining the fitted surface via contour plots (Lawson and Erjavec, 2001). A central composite design (CCD) is one of the most commonly used RSM for fitting second order models (Ferreira, 2009). Central composite design consists of factorial points, axial points or star points, and center points. The factorial points allow the estimation of all the main effects and the factor interactions. The axial points allow the determination of all the quadratic terms. Center points provide a check of the adequacy of the model prediction (Lawson and Erjavec, 2001). In this study a CCD was used to optimize the enzymatic hydrolysis of SBP. Three independent variables (SBP percent solid, enzyme concentration, and hydrolysis temperature) were studied. Table 1 shows the effects of each variable

Table 1. Experimental range and levels of the independent variables.

Variables	-1.68	-1	0	+1	+1.68
X1 Temperature (°C)	33	40	50	60	67
X2 Enzyme loading (mg/g cellulose)	6.6	10	15	20	24
X3 SBP percent solid (%)	0.66	1	1.5	2	2.34

studied and their subsequent levels. The unit of the enzyme loading (X_2) is in mg of enzymes per gram of cellulose in the biomass. Alpha is the distance from the center to the axial points. The value of alpha is determined by the rotatability. Rotatability implies that the accuracy of predictions from the quadratic equation only depends on how far away from the origin the point is, not the direction (Lawson and Erjavec, 2001). For three factors, rotatability gives an alpha (α) value of 1.68. The relation between the coded values and the actual values is described by the equation below where X_i is the coded value, X_a is the actual value, X_o is the center point, and X_h is the high value. The below equation converts the coded values given by Minitab 15 to actual values. The equation was obtained from Lawson and Erjavec, (2001).

$$X_i = \frac{X_a - X_o}{X_h - X_o}$$

The experimental design is presented in Table 2. The experimental design constituted a total of 20 runs, 2^3 factorial points, 6 star points, and 6 center points. Each point was replicated for a total of 40 runs. First, a 2^3 factorial was run with a few center points. A curvature test was performed and found to be significant. As a result, the alpha points and additional center points were run. Upon completion of the experiments, glucose concentration was measured as the response. The percent hydrolysis yields for the total runs are given as Y_1 and Y_2 in Table 2.

The mathematical relationship between the response of the variables and the independent variables can be presented by a second-degree quadratic polynomial equation where Y is the predicted response, X_1, X_2, X_3 are the independent variables, b_o is the constant, b_1, b_2, and b_3 are the linear coefficients, b_{11}, b_{22}, and b_{33} are the squared effects terms, b_{12}, b_{13}, and b_{23} are the cross-product interaction terms. All the statistical calculations performed on the data were accomplished using Minitab 15 software.

$$Y = b_o + b_1 \bullet X_1 + b_2 \bullet X_2 = b_3 \bullet X_3 = b_{11}\ X_1^2 + b_{22} \bullet X_2^2$$

$$b_{33} \bullet X_3^2 = b_{12} \bullet X_1 \bullet X_2 + b_{13} \bullet X_1 \bullet X_3 + b_{23} \bullet X_2 \bullet X_3$$

Table 2. Central Composite Design Matrix for Three Independent Variables on the Glucose Yield and Experimental Results.

Run No.	Reaction Temp. (°C)	Enzyme Loading (mg/g Cellulose)	SBP Loading (%)	Glucose Yield (%) Y_1	Y_2
1	40	10	1.0	50.0	48.0
2	60	10	2.0	48.0	47.4
3	67	15	1.5	41.0	39.0
4	50	24	1.5	69.1	74.6
5	60	20	1.0	43.1	39.1
6	40	20	2.0	78.0	73.0
7	40	20	1.0	72.4	68.0
8	60	20	2.0	56.2	52.1
9	60	10	1.0	23.5	24.2
10	40	10	2.0	48.2	46.3
11	50	6.6	1.5	65.0	58.0
12	50	15	0.66	85.4	84.3
13	33	15	1.5	65.0	62.0
14	50	15	2.3	76.6	75.6
15	50	15	1.5	77.0	78.0
16	50	15	1.5	79.4	78.0
17	50	15	1.5	81.3	83.0
18	50	15	1.5	81.9	77.0
19	50	15	1.5	78.0	79.0
20	50	15	1.5	82.0	82.0

RESULTS AND DISCUSSION

The optimization of enzymatic hydrolysis of SBP using a central composite design was studied. The experimental values of hydrolysis yield given in Table 2 were subjected to multiple regression analysis using Minitab 15 software. The results of the multiple regression analyses are presented in Table 3 along with t-values and the p-values. The student's t-test and p-value were performed to determine significance of the regression coefficients. The size of the regression coefficients for each independent variable gives the size of the effect that variable has on the response. Coefficients with negative signs

Table 3. Estimated regression coefficient of the second order polynomial model.

Coefficient	Estimated Coefficient	t-value	P-value
b_0	-331.53	-5.56	<0.001
b_1	12.91	8.07	<0.001
b_2	13.78	5.04	<0.001
b_3	-5.86	-0.21	0.835
b_{11}	-0.14	-10.26	<0.001
b_{22}	-0.301	-5.69	<0.001
b_{33}	-9.49	-1.69	0.102
b_{12}	-0.062	-1.65	0.110
b_{13}	0.8	2.13	0.042
b_{23}	-0.118	-0.16	0.877

suggest that there is a negative effect on the yield. The larger the t-value and the smaller the p-value ($p<0.05$) are indications of the significance of the coefficient and the effects on the hydrolysis process. A p-value of less than 0.05 was used as our model criteria, meaning we are at least 95% confident that one or more effects for the factors are nonzero.

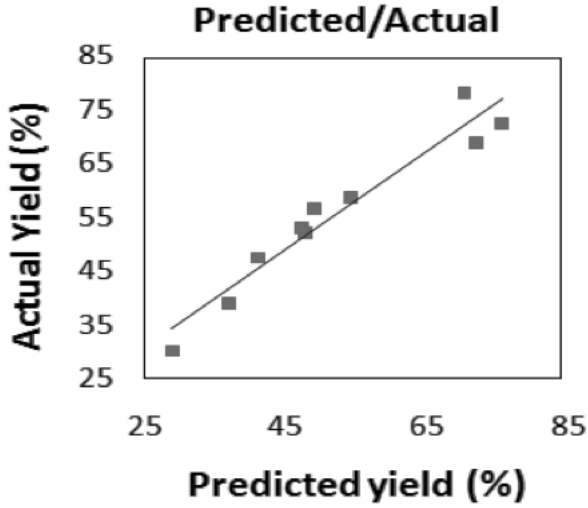
The reaction temperature (X_1) and enzyme loading (X_2) were found to have significant effects on the hydrolysis yield. Sugar beet pulp percent solid (X_3) was found to be not significant. The square terms for reaction temperature and enzyme concentration were found to be significant and have negative effect on the hydrolysis yield. However, the interaction terms were not significant; meaning the effects of one factor is not dependent on the setting of the other factors. The variables, which were not significant, were eliminated from the model one at a time starting with coefficient with the smallest t-value and the regression analysis was repeated. The empirical relationship between the hydrolysis yield and the studied variables in uncoded units is given below

$$\mathbf{Y = -308.7 = 12.9X_1 + 10.2X_2 - 0.139X_1^2 - 0.291X_2^2}$$

A plot of predicted percent yield versus actual percent yield is presented in Figure 1. The model is in good agreement with the actual experimental yields. From the graph (Figure 1) it is evident that the model is in concord with the experimental data.

To determine the statistical significance of the model, an analysis of variance was performed at the 95% confidence level. Table 4 contains the results of the analysis of variance. The model fit well with

Figure 1. Plot of actual percent yield versus predicted percent yield.



p-value of <0.001. The linear terms and the square terms were also significant with p-value of <0.001. The model fit (R-square) was determined to be 0.82. Joglekar and May reported that an R-squared of 0.80 is acceptable (Joglekar and May, 1987). Lack of fit was found to be significant with a p-value of <0.001. However, the high R-square value (0.82) suggests that the model is adequate in predicting the relationship between the variables and the response. Therefore, the model is appropriate to predict hydrolysis yield.

The polynomial equation is graphically presented in the surface plot and contour plot. Figure 2 shows the response surface plot of the polynomial as a function of reaction temperature and enzyme load-

Table 4. Analysis of variance of second-order polynomial model.

Source	DF	Seq SS	F-test	P-value
Regression	4	10822	40.0	<0.001
Linear	2	3879	48.7	<0.001
Square	2	6943	51.0	<0.001
Residual Error	35	2368		
Lack-of-fit	4	1438	12	<0.001
Pure Error	31	930		
Total	39	13190		
R^2=0.82				

Figure 2. Response surface plot of the polynomial as a function of the reaction temperature and enzyme loading.

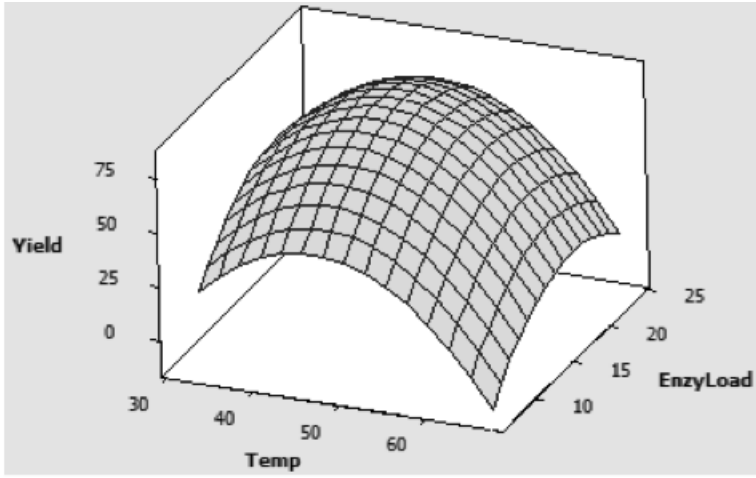


Figure 3. Contour plot of the combine effect of enzyme loading and reaction temperature.

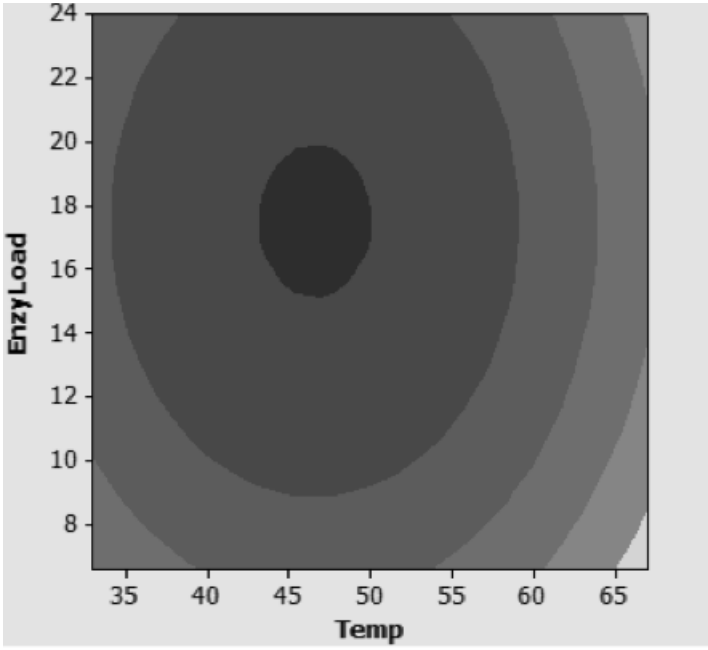


Table 5. Confirmation experimental design.

Run No.	Reaction Temp. (°C)	Enzyme Loading (mg/g Cellulose)	SBP Loading (%)	Y (actual) (%)	Y (predicted) (%)	Error limit (%)
1	46	17.6	1.5	82	85	±5.6
2	50	24	1.5	72	69	±8.3
3	60	10	1.0	36	40	±6.6

ing. The plot allows the effects of the parameters on the response to be determined. Reaction temperature is considered to be one of the major factors affecting hydrolysis yield (Schell et. al, 1999). Hydrolysis yield increased initially, but decreased with higher reaction temperature. This is in agreement with the enzyme vendor's recommendation. The optimal temperature of the enzyme is 50°C. Thus, carrying on reactions at higher temperature would render the enzyme ineffective. Hydrolysis yield increased with increasing enzyme loading from low levels to the center (15 mg/g), and decreased with high enzyme loading.

A contour plot of the effects of enzyme loading and reaction temperature on hydrolysis yields is shown in Figure 3. It can be seen from Figure 3 that high hydrolysis yields can be obtained by keeping the reaction temperature and the enzyme loading at the center.

Confirmation Experiments

To validate the model, three confirmation experiments were performed. The result of the conformation experiments are presented in Table 5. Table 5 contains additional experiments, along with the actual yields, predicted yields, and error limits on the predictions. The first run is the predicted optimal condition. The last two conditions for the confirmation experiment were among the conditions presented in Table 2.

The experimental results show that with enzyme concentrations of 17.6 mg/g, reaction temperature of 46°C, SBP percent solid of 1.5% (Because the SBP solid was not found to be significant from the model, the center point of 1.5% was chosen to validate the model.) and reaction time of 72 h, a glucose yield of 82% was achieved. The model predicted yield was 85 ± 5.6%. The achieved actual yield is within the indicated error limit. Therefore, the model is adequate in predicting the yield and optimizing the enzymatic hydrolysis of SBP.

CONCLUSIONS

Enzymatic hydrolysis is the second most expensive step in ethanol production (Wooley et. al, 1999). Therefore, optimizing this process would contribute to commercialization of lignocellulosic ethanol. For

that reason, we set out to develop a mathematical model to predict glucose yields and to optimize the enzymatic hydrolysis of cellulose in sugar beet pulp. A central composite design was performed to investigate the effects of enzyme loading, reaction temperature, and SBP percent solid on the hydrolysis yield. The reaction temperature and enzyme concentration were found to have a significant effect on the hydrolysis yield. Sugar beet pulp percent solid was found to be insignificant. The interactions terms were found to be insignificant. The experimental yield of 82% was in agreement with the predicted yield of 85 ± 5.6%, which validated that the model is adequate in predicting hydrolysis yield.

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