OPTIMIZATION OF ENZYME DOSAGES FOR HYDROLYSIS OF DESTARCHED CORN FIBER SUBJECTED TO ACID AND ALKALINE PRETREATMENTS FOR IMPROVED FERMENTABLE SUGAR YIELD

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Optimization of enzyme dosages for the hydrolysis of acid and alkaline treated destarched corn fiber with the objective of increasing the fermentable sugar yield was investigated using response surface methodology. Destarched corn fiber was pretreated by 0.75% of sulfuric acid and sodium hydroxide in the autoclave at 121°C for 1 h. The dry solid fraction was used for determining the composition, for characterization (crystallinity index, microstructure), and enzymatic saccharification. The experimental design for enzyme dosage optimization had fifteen combinations with three levels (-1, 0, 1) of Celluclast 1.5L, β-glucosidase and Viscozyme L. When compared to acid pretreated fiber, alkaline treated destarched corn fiber presented significantly decreased lignin content for efficient enzymatic saccharification. The optimum enzyme dosages for alkaline treated destarched corn fiber were as follows: 6.78 and 6.05 FPU/g of Celluclast 1.5L, 28.65 and 30.18 CBU/g of β-glucosidase, and 2.31 and 0.90 FBG/g of Viscozyme L, which allowed obtaining maximum reducing sugar (316.20 mg/g) and glucose (6.09 mg/g).

Keywords: response surface methodology, saccharification, destarched corn fiber, acid and alkaline pretreatments

INTRODUCTION

Corn fiber represents a renewable resource that is available in sufficient quantities from the corn wet-milling industries to serve as a low-cost feedstock for ethanol production. It contains approximately 17% residual starch, 18% cellulose and 35% hemicelluloses.[1] Efficient and economical technologies for lignocellulosic ethanol production will play a major role in the conversion of biomass and its success depends largely on the development of environment-friendly pretreatments, highly effective enzymes for the conversion of pretreated corn fiber to fermentable sugars, and efficient microorganisms for fermentation. In their native conformation, cellulose and hemicelluloses are largely protected from enzymatic degradation due to associations of these polymers with lignin and with each other, which act as a barrier and interfere with hydrolysis.[2,3] Therefore, pretreatment is required to disrupt the structure of lignocellulosic materials to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars. The extensive interactions among cellulose, hemicellulose and lignin, and the barrier nature of lignin minimize enzyme access to the carbohydrates and result in poor yields of fermentable sugars.[4,5]

The production of bioethanol from renewable biomass is also affected by the composition and structure of the feedstock, pretreatment method, type and loading of enzymes, cellulose crystallinity, and available surface area.[6]The crystalline structure of cellulose limits the available sites for enzymatic attack as the average size of the capillaries in biomass is too small to allow entry to large enzyme molecules. Enzymatic action is confined to the external surfaces unless the feedstock structure is modified.[7] Dilute acid pretreatment has been developed for hydrolyzing lignocellulosic biomass for fermentable sugar production. Alkaline pretreatment removes acetyl and various uronic acid substitutions on hemicelluloses, which reduce the accessibility of hemicelluloses and cellulose to the enzymes.[8]However, sulfuric acid pretreatment involves the corrosion of materials

and the incorporation of salts into the biomass during the pretreatment reactions. A significant disadvantage of alkaline pretreatment is the conversion of alkali into irrecoverable salts and/or the incorporation of salts into the biomass during pretreatment reactions. Therefore, using a large amount of alkali becomes a challenging issue for the pretreatment.[9] Enzyme dosage also significantly affects the cost of the overall ethanol production process. Enzyme concentration beyond a certain level led to a decrease in the amount of reducing sugars liberated. Faster sugar production with an enzyme overdose might lead to inhibition of the hydrolysis process.[10]

The optimization of enzyme dosages for fermentable sugar yield is one of the most important stages in the development of an efficient and economical ethanol production. The traditional 'one-factor-at-a-time approach' is time-consuming, moreover, the interactions between independent variables are not considered. Response surface methodology (RSM) is an effective optimization tool wherein many factors and their interactions affecting the response can be identified with fewer experimental trials. RSM has been widely used in various fields ranging from food process operations, including extrusion, new product development, in biotechnology – for medium composition, to bioprocessing, such as enzymatic hydrolysis and fermentation.[11,12] Therefore, the present study was conducted to investigate the effect of chemical pretreatments on lignocellulosic composition, crystallinity and microstructure for enzymatic saccharification, and to determine the optimum enzyme dosage for achieving maximum fermentable sugar yield of destarched corn fiber.

EXPERIMENTAL

Materials

Corn fiber provided by Samyang Genex Co., Korea was used as substrate sample. Celluclast 1.5L and ViscozymeL were obtained from Novozyme Co. Ltd (Bagsvaerd, Denmark). β-glucosidase was purchased from Sigma Co. Ltd (UK).The other necessary chemicals and reagents were purchased from Aldrich, St. Louis, MO.

Preparation of the substrate

A schematic diagram for destarching of corn fiber, chemical pretreatments, and enzymatic saccharification is presented in Figure 1. The starch contained in corn fiber (CF) was eliminated according to the method of Gaspar *et al.*[13] One kilogram of corn fiber was suspended in 10 L of distilled water and the pH was adjusted to 5.3-5.6 by using 6 N NaOH. Liquozyme supra from Donghee Co. Ltd., Korea (10% v/w) was added and reacted at 121°C for 1 h. The solid residue, destarched corn fiber (DCF) was washed with DH_2O to pH 7and dried at 50°C for 24 h.

Chemical pretreatments

Destarched corn fiber (10% w/w) was added to 0.75% (v/v) of sulfuric acid[14] and sodium hydroxide[15] at 121°C for 1 h. DCF without any chemical pretreatment was also included as control. The mixture was neutralized with 5 N NaOH and 37% (v/v) HCl and filtered to separate residues and the filtrate fraction.

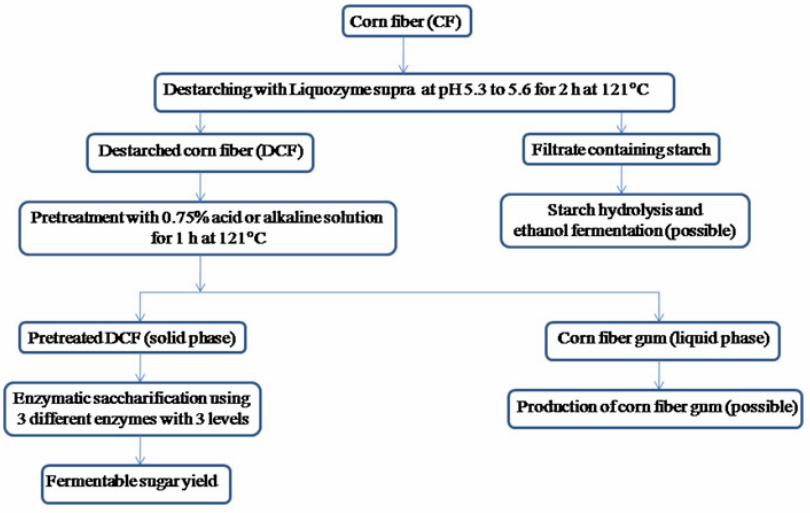

Figure 1: Schematic diagram for destaching of corn fiber, chemical pretreatments and enzymatic saccharification process

The solid residues were dried at 50°C and used for the analysis of lignocellulosic composition, crystallinity index, microstructures and saccharification process.

Lignocellulosic composition

Cellulose content was determined by the nitric-acetic acid method.[16] Cellulose content was calculated as a percentage of starting material. Hemicelluloses content was determined according to the phloroglucinol method with xylose as a standard.[17] One hundred milligram of samples were weighed into screw cap test tubes (30 x 110 mm) and suspended in 1.5 mL of sodium acetate buffer (50 mM, pH 5.0). The tubes were incubated in water bath (150 rpm) at 50°C for 20 min. After exactly 20 min, the tubes were plunged into boiling water for 2 min. After cooling, the slurry was centrifuged at 3000 rpm for 30 min. Two hundred microliters of supernatant was mixed with 1 mL of freshly prepared phloroglucinol reagent (glacial acetic acid, concentrated hydrochloric acid, 20% (w/v) phloroglucinol in ethanol, and 1.75% (w/v) glucose in water). The tubes were capped and boiled for 40 min. After cooling rapidly in cold water, the absorbance at 552 and 510 nm were recorded. The difference between the absorbance at 552 and 510 nm was used to calculate xylose equivalents in solution. The hemicelluloses content was calculated by using the following equations.[18]

Hemicelluloses content (%) = (132/150) ×(C) × (V/M) ×100

where 132/150 is the stoichiometric factor between xylose and xylan, C is the xylose content (g/L), V is the total volume of sugar solution (L) and M is the weight of the sample (g).

Lignin content was determined as the residue remaining after total hydrolysis of cell-wall polysaccharides described by Van Eylen *et al.*[19]

Crystallinity index

X-ray diffraction of pretreated DCF was determined according to Jin *et al.*[20] The scan was carried out in an X-ray diffractor (RIGAGU# 0/M AX-2500, Japan). The measurement conditions were CuKa (1.54 nm), 40kV, 100mA, 2θ=10-40°, scan rate 10°/min, reflection model. Pure cellulose was used as reference, with the diffraction peak at 2θ=23° corresponding region.[21] The intensity of the diffraction peak at 2θ=23° for all sample was a measure of their relative crystallinity, and this was used to compare acid and alkaline treated DCF for structural modification.

The crystallinity index (CI) was calculated using the following equation:[22]

$CI\ (\%) = (I_p - I_{am})/I_p \times 100$

where I_p is the peak intensity and I_{am} is the intensity attributed to amorphous cellulose.

Microstructures

The microstructure of DCF after chemical pretreatment was examined with a field emission scanning electronic microscope (MIRA II LMH Tescan USA, Inc., Cranberry Township, PA). The samples were fixed in stubs containing a gold-palladium alloy before observation. All samples were examined using at an accelerated voltage of 10 kV.

Enzyme cocktail optimization

A combination of cellulase (Celluclast 1.5L), β-glucosidase and cell-wall degrading enzyme complex (Viscozyme L) was used for enzymatic saccharification. The enzyme activity of Celluclast 1.5L was 70 FPU/g with an optimal pH range between 4.5-6.0 and an optimal temperature range of 50-60°C, β-glucosidase with enzyme activity of 322 CBU (cellobiase unit)/mL was used to hydrolyze cellobiose. Viscozyme L (Novozymes) contained a wide range of carbohydrases, including arabinase, cellulase, β-glucanase, hemicellulase and xylanase, which act on branched pectin-linked substances found in plant cell walls. Its activity was 13.4 FBG (fungal beta-glucanase unit)/mL.

Enzymatic saccharification

Three grams of sample was saccharified in the rotary shaker (150 rpm) at 45°C for 72 h by using three levels of three different enzymes per gram of DCF in 50 mL of 0.05 M sodium acetate-acetic acid buffer (pH 4.8). Based on the previous study, the three levels of three different enzymes (Table 2) were selected.[23] Three levels of the three different enzymes used in this experiment were selected, corresponding to the concentrations of 0.0254, 0.0609 and 0.1016mL/g of DCF. These concentrations are equivalent to 1.78, 4.26 and 7.11 FPU of Celluclast 1.5L, 8.18, 19.61 and 32.72 CBU of β-glucosidase, and 0.34, 0.82 and 1.36 FBG of Viscozyme L, respectively. After saccharification, the reducing sugar yield was determined according to the DNS method[24] with 3.5-dinitrosalicilic acid. The glucose content was measured by using glucose oxidase-peroxidase assay kit.

Experimental design and statistical analysis

Response surface methodology (RSM) was used to determine the effects of the dosage ofCelluclast 1.5L, β-glucosidase and Viscozyme L on the reducing sugarand glucose yields of acid and alkaline treated DCF. The central composite experimental design described by Box and Draper[25] for three variables with three levels of each variable was used. The data were analyzed using a response surface regression procedure (SAS Institute, Cary, NC). The generalized equation model is the following:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1X_1+b_{22}X_2X_2+b_{33}X_3X_3$$

where Y= objective response, X_1= Celluclast 1.5L (FPU/g), X_2= β-glucosidase (CBU/g), X_3= Viscozyme (FBG/g), b_0= intercept, b_n= regression coefficient.

For each response, three dimensional plots were produced from the regression equations by holding the other variable at a fixed center point. If a stationary point was a saddle point, the maximum or minimum response was obtained using ridge analysis.

Verification of the models

Optimum enzyme dosages for reducing sugar and glucose yields from alkaline treated DCF dependent on enzyme dosages of Celluclast 1.5L, β-glucosidase and Viscozyme L were obtained using prediction equations of RSM. The models were reconfirmed by three replications under the optimum enzyme dosages for alkaline treated DCF. The experimental and predicted values were compared in order to determine the validity of the models.

RESULTS AND DISCUSSION

Starch removal and lignocellulosic composition

After destarching corn fiber with Liquozyme supra, most of the hydrolyzed starch (99.51%) was present in the supernatant and only a negligible amount of it (0.49%) remained in the solid DCF. DCF was pretreated with 0.75% of sulfuric acid and sodium hydroxide solutions. The lignocellulosic composition of CF, DCF and acid and alkaline treated DCF is shown in Table 1. After acid pretreatment, most of the hemicellulose dissolved out into the liquid fraction, while cellulose remained in the solid fraction. It was also confirmed by total solid recovery of pretreated DCF. The high recovery of cellulose may be due to the removal of hemicellulose from acid treated DCF. Saha *et al.*[14] also reported that dilute acid pretreatment removes hemicelluloses and reduces the need for use of hemicellulase enzymes for degrading of biomass. The action mode of dilute acid is to solubilize hemicelluloses and leave lignin and cellulose intact, so that the enzymatic digestibility of cellulose is enhanced.

Table 1
Starch removal and lignocellulosic composition of solid fraction (g/100 g, db) of acid and alkaline treated destarched corn fiber (DCF)

Solid sample	Starch	Solid residue	Cellulose	Hemicellulose	Lignin
[a]CF	17.40	-	19.69[d]	20.28[b]	21.42[d]
DCF	0.49	-	26.74[a]	29.76[a]	28.78[a]
DCF (Control)	-	97.73[a]	20.68[c]	30.31[a]	23.67[c]
DCF (0.75% H_2SO_4)	-	52.19[c]	24.49[b]	12.46[c]	27.25[b]
DCF (0.75%NaOH)	-	54.44[b]	18.98[d]	27.27[a]	21.80[d]

[a]Corn fiber. Means of three replications based on the least significant difference procedure at α = 0.05 level. Means with the same letter in the same column are not significantly different

Table 2
X-ray diffraction results (corresponding to 2θ and maximum intensity) and crystallinity index of acid and alkaline treated destarched corn fiber (DCF)

Solid sample	2θ	Maximum intensity (linear count per second)	Crystallinity index (%)
Pure cellulose	23	1159	82.74
DCF (Control)	20.48	191.13	65.10[b]
DCF (0.75% H_2SO_4)	20.30	230.67	68.67[a]
DCF (0.75%NaOH)	17.76	201.73	70.52[a]

Means of three replications based on the least significant difference procedure at α = 0.05 level. Means with the same letter in the same column are not significantly different

However, alkaline treated DCF had a significantly increased hemicellulose content, compared to that of the acid treated one. Alkaline-based methods are generally more effective at solubilizing a greater fraction of lignin, while leaving behind much of hemicelluloses in an insoluble polymeric form.[26,27] Some studies observed that the removal of hemicellulose increases the accessibility of cellulose to become hydrolyzed.[28] In this case, enzymatic requirements

for hemicellulosic modification for higher fermentable sugar production must be taken into consideration.[29] Alkaline pretreatment offers good performance in terms of recovering hemicellulose with an increase in hemicellulosic sugar recovery. Therefore, a significant increase in the hemicellulose fractions would not have a major impact on the enzymatic hydrolysis and the improvement in enzymatic hydrolysis is not the result of the removal of hemicellulose alone.[30]

The lignin content of alkaline treated DCF was significantly decreased compared to those of the control and acid treated ones, because of the solubilization of lignin in the alkaline solution. The cellulose and hemicelluloses are cemented together by lignin. Lignin limits the rate and extent of enzymatic hydrolysis by acting as a shield, preventing the digestible parts of the substrate to be hydrolyzed.[8] Alkaline pretreatment of lignocellulosic substances of corn fiber disrupts the cell wall by dissolving lignin, hydrolyzing uronic and acetic acid esters and swelling cellulose,[31] and also increases the biodegradability of the cell wall due to cleavage of the bonds between lignin and cellulose.[32]

The main explanation of the improvement in enzymatic hydrolysis after the removal of lignin is related to the accessible surface area of cellulose. The effect of this area may correlate with the crystallinity index or lignin protection or both of them. Lignocellulosic materials have external and internal surface area. The external surface area is related to the size and shape of the particles, while the internal surface area depends on the capillary structures of cellulosic fibers. Alkaline pretreatment of lignocellulosic materials causes swelling, leading to an increase in internal surface area, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structures.[33]

Crystallinity index

The crystallinity index of pretreated DCF is presented in Table 2.There was no significant decrease in the crystallinity index among pretreated DCF. On the other hand, the crystallinity index of pretreated DCF was significantly increased ($p<0.05$) compared to that of the untreated one. The increase in the crystallinity index of acid treated DCF may be due to the increase of the cellulose fraction after the pretreatment (Table 1). An increase in the cellulose crystallinity index using NaOH has also been observed previously.[34,35,36,37] This fact has been suggested to be due to not only the removal or reduction of more easily available amorphous cellulose, but also the delignificationof DCF[35] (Table 1). It was clear that the mechanism of alkaline pretreatment rendered the substrate more susceptible to enzymatic hydrolysis. This result was also confirmed by the reducing sugar and glucose yields of alkaline treated DCF (Table 3). Lee *et al*.[38] suggested that opening of the cell wall structure at the microscopic scale due to chemical pretreatment would be sufficient for enzymatic saccharification, regardless of the crystallinity index. Yoshida *et al*.[35] also found that delignification increased the rate of enzymatic hydrolysis of cellulose and hemicelluloses, although biomass showed higher crystallinity index. The present results are in agreement with those of their observations.

Microstructures

The surface morphology of DCF without pretreatment was observed to be smooth and compact. A compact structure or fewer pores on the surface of the product can cause slower migration or water penetration during hydrolysis. DCF treated by acid and alkaline showed changes in the surface structure with extensive serration, tunneling and surface erosion, which can probably cause a decrease of hardness. These structural changes make acid and alkaline treated DCF more susceptible to enzymatic digestion. However, alkaline treated DCF has a more open structure and a high degree of porosity (Fig. 2), which was confirmed by the high water solubility index of the substrate (data not shown).

The mechanism of alkaline pretreatment consists in the saponification of intermolecular ester bonds crosslinking, and the porosity of lignocellulosic materials increases with the removal of the crosslinks.[39] A porous structure would facilitate rapid water diffusion or promote water uptake during hydrolysis. The size and number of the pores can significantly influence the texture of the substrate. A smaller number of pores and small sizes lead to a dense structure, while a larger number of pores and large pores can cause a decrease in hardness of the substrate. Therefore, alkaline treatment of DCF resulted in the disruption of the fiber structure for efficient enzymatic saccharification and increased the reducing sugar and glucose yields (Table 3).

Table 3
Central composite design with reducing sugar and glucose yields (mg g^{-1}, db) of acid and alkaline treated destarched corn fiber (DCF)

	-1	0	1	DCF (Control)				DCF (0.75% H_2SO_4)				DCF (0.75%NaOH)			
X_1Celluclast 1.5L	1.78	4.26	7.11 (FPU/g)												
X_2 β-glucosidase	8.18	19.61	32.72 (CBU/g)												
X_3Viscozyme L	0.34	0.82	1.36 (FBG/g)												
Test No.	X_1	X_2	X_3	[a]ORS	[b]PRS	[c]OG	[d]PG	[a]RS	[b]PRS	[c]OG	[d]PG	[a]ORS	[b]PRS	[c]OG	[d]PG
1	-1	-1	0	82.21	85.29	2.34	2.14	108.49	107.72	3.17	3.29	149.71	151.46	3.71	3.43
2	1	-1	0	111.76	107.61	3.98	4.17	158.41	161.35	2.42	2.65	287.58	273.52	5.68	5.51
3	-1	1	0	84.48	88.24	3.16	2.99	113.30	110.45	3.27	3.03	181.49	195.34	4.71	4.92
4	1	1	0	110.43	107.75	3.76	3.93	208.77	209.43	2.16	2.05	303.22	301.69	6.84	7.09
5	-1	0	-1	81.93	82.04	2.56	2.50	116.05	115.62	3.02	3.19	179.31	165.41	3.89	4.09
6	1	0	-1	104.73	111.58	3.69	3.28	197.67	193.71	2.46	2.48	286.59	289.92	6.54	6.65
7	-1	0	1	115.80	108.86	2.19	1.61	125.57	129.61	3.16	3.12	203.97	202.28	5.16	5.03
8	1	0	1	120.30	120.28	3.93	3.97	200.27	200.61	2.38	2.23	293.77	306.03	6.83	6.66
9	0	-1	-1	84.68	81.54	3.22	3.47	123.14	124.33	3.33	3.04	204.60	216.77	4.79	4.87
10	0	1	-1	91.11	87.30	3.57	3.78	137.63	140.82	2.63	2.73	253.54	251.94	6.96	6.58
11	0	-1	1	99.79	104.01	3.54	3.29	131.13	127.76	3.06	3.00	242.44	242.59	5.18	5.55
12	0	1	1	98.28	101.01	3.89	3.67	160.85	159.84	2.20	2.46	291.46	280.74	6.95	6.88
13	0	0	0	99.03	100.14	3.50	3.55	127.37	131.91	2.97	2.95	263.04	260.03	5.75	5.82
14	0	0	0	99.86	100.14	3.50	3.55	129.90	131.91	2.97	2.95	258.88	260.03	6.03	5.82
15	0	0	0	101.53	100.14	3.50	3.55	128.46	131.91	2.89	2.95	258.18	260.03	5.67	5.82

[a]Observed reducing sugar, [b]Predicted reducing sugar, [c]Observed glucose, [d]Predicted glucose

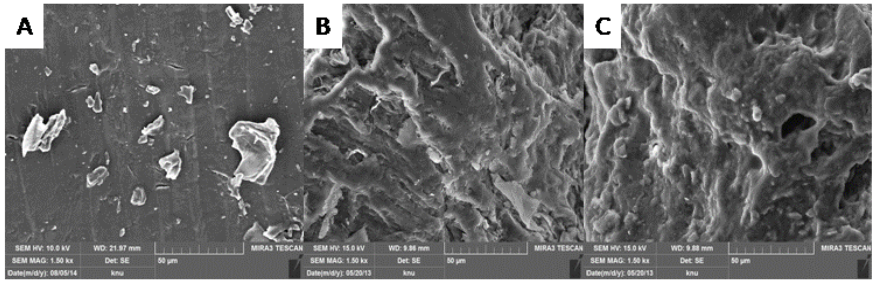

Figure 2: Microstructures of acid and alkaline treated destarched corn fiber (DCF); A – DCF (control); B – DCF (0.75% H_2SO_4); C – DCF (0.75% NaOH)

Table 4
Regression coefficients of predicted quadratic polynomial models for reducing sugar and glucose yields of acid and alkaline treated destarched corn fiber (DCF)

Coefficient	DCF (Control)		DCF (0.75% H_2SO_4)		DCF (0.75%NaOH)	
	Reducing sugar	Glucose	Reducing sugar	Glucose	Reducing sugar	Glucose
b_o	38.92	1.03	137.61***	2.96**	-5.63	0.43
Linear						
b_1	2.15	0.88**	-11.71**	0.05	55.03***	1.21***
b_2	2.53*	-0.01	0.09	0.02	5.31*	0.12
b_3	36.68	0.08	-56.31**	0.60	51.16	-0.10
Cross product						
b_{12}	-0.02	-0.01	0.35***	-0.003	-0.12	0.0007
b_{13}	-3.33	0.29*	-1.31	-0.03	-3.82	-0.17
b_{23}	-0.35	0.002	0.62	-0.001	0.12	-0.02
Quadratic						
b_{11}	0.56	-0.08**	2.25***	-0.01	-3.15**	-0.08**
b_{22}	-0.05*	0.001	-0.03	-0.0004	-0.08	-0.001
b_{33}	1.42	-0.89	35.38**	-0.25	-6.20	0.96
R^2	0.9076	0.91	0.99	0.84	0.97	0.96
Probability of *F*	0.04	0.04	0.0001	0.13	0.003	0.006
C.V.	6.32	11.35	3.37	9.50	5.89	6.54

Model by which X_1(Celluclast 1.5L), X_2 (β-glucosidase), X_3(Viscozyme L) was calculated: $Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1X_1+b_{22}X_2X_2+b_{33}X_3X_3$

Table 5
Analysis of variance for the response surface quadratic model for reducing sugar and glucose yields of acid and alkaline treated destarched corn fiber (DCF)

Pretreatment	Sugar	Residual	[a]DF	[b]SS	[c]MS	F-value	Probability (p) > F
DCF (Control)	Reducing sugar	Lack of fit	3	192.81	64.27	39.71	0.025
		Pure error	2	3.24	1.62		
		Total error	5	196.05	39.21		
	Glucose	Lack of fit	3	0.70	0.23	89.96	0.011
		Pure error	2	0.005	0.003		
		Total error	5	0.70	0.14		
DCF (0.75% H_2SO_4)	Reducing sugar	Lack of fit	3	74.09	24.70	1.07	0.514[d]
		Pure error	2	45.82	22.91		
		Total error	5	119.91	23.98		
	Glucose	Lack of fit	3	0.35	0.12	51.68	0.019
		Pure error	2	0.005	0.002		
		Total error	5	0.36	0.07		
DCF (0.75%NaOH)	Reducing sugar	Lack of fit	3	1018	339.41	49.02	0.201[d]
		Pure error	2	13.82	6.91		
		Total error	5	1032	206.41		
	Glucose	Lack of fit	3	0.61	0.20	5.68	0.153[d]
		Pure error	2	0.07	0.04		
		Total error	5	0.68	0.14		

[a]Degree of freedom, [b]Sum of squares, [c]Mean square, [d]Insignifican

Predicted models and statistical analysis

Multiple regression equations were generated relating response variables to coded levels of the independent variables. Multiple regression coefficients were determined by employing to predict quadratic polynomial models for reducing sugar and glucose yields.[25] Table 3 summarizes the data for reducing sugar and glucose yields of acid and alkaline treated DCF.

The effects of enzyme dosage variables: Celluclast 1.5L, β-glucosidase and Viscozyme L on reducing sugar and glucose yields of DCF were regressed and presented in Table 4. Reducing sugar and glucose yields of acid treated DCF were lower than those of the alkaline treated one. Zhao *et al*.[40] reported that alkaline (NaOH) pretreatment could lead to a higher enzymatic conversion ratio of cellulose compared with sulfuric acid pretreatment. Compared with acid pretreatment, alkaline pretreatment appears to be the most effective method in breaking the ester bonds between lignin, hemicellulose and cellulose.[13]

The goodness of fit of the models was checked by the coefficient of determination (R^2). Guan and Yao[41] reported that R^2 should be at least 0.80 for the good fit of a model. R^2 for reducing sugar and glucose yields of alkaline treated DCF was found to be 96.87 and 95.77% with *p*-value coefficients of 0.0030 and 0.0062 respectively, which implied that the models proved suitable for the adequate representation of the actual relationship between the selected variables.[42] These values indicated that the regressions are statistically significant and only 0.04% of the total variation was not explained by the regression models. The lack of fit of *F*-value and *p*-value for reducing sugar and glucose yields of alkaline treated DCF were 49.124 and 0.201, and 5.678 and 0.153, respectively (Table 5). These values indicated that they were not significant relative to the pure error.[42]

Table 6

Analysis of variance of the regression parameters of the predicted response surface quadratic model for reducing sugar and glucose yields of acid and alkaline treated destarched corn fiber (DCF)

Pretreatment	Sugar	Regression	[a]DF	[b]SS	Coefficient (R^2)	F-value
DCF (Control)	Reducing sugar	Linear	3	1531	0.72	13.01***
		Quadratic	3	291	0.14	2.48
		Cross product	3	103	0.05	0.88
		Total	9	1925	0.91	5.46**
	Glucose	Linear	3	4.73	0.60	11.23**
		Quadratic	3	1.47	0.19	3.48
		Cross product	3	0.93	0.12	2.21
		Total	9	7.12	0.91	5.64**
DCF (0.75% H_2SO_4)	Reducing sugar	Linear	3	13163	0.87	183.0***
		Quadratic	3	1290	0.09	17.94***
		Cross product	3	590	0.04	8.20**
		Total	9	15044	0.99	69.70***
	Glucose	Linear	3	1.72	0.79	8.08**
		Quadratic	3	0.05	0.02	0.24
		Cross product	3	0.05	0.02	0.24
		Total	9	1.83	0.84	2.85
DCF (0.75%NaOH)	Reducing sugar	Linear	3	29501	0.89	47.64***
		Quadratic	3	2259	0.07	3.65*
		Cross product	3	172	0.01	0.28
		Total	9	31933	0.97	17.19***
	Glucose	Linear	3	13.69	0.85	33.49***
		Quadratic	3	1.47	0.09	3.60
		Cross product	3	0.26	0.02	0.63
		Total	9	15.42	0.96	12.58***

[a]Degree of freedom, [b]Sum of squares; ***,** and * indicate significance at $p < 0.01$, and 0.05 and 0.1, respectively

Table 7
Analysis of variance of the factors and the critical values obtained from response surface for reducing sugar and glucose yields of acid and alkaline treated destarched corn fiber (DCF)

Pretreatment	Sugar	Enzymes	Analysis of variance				Criticalvalue	
			[a]DF	[b]SS	[c]MS	F value	Coded	Uncoded
DCF (Control)	Reducingsugar	Celluclast 1.5L	4	1021	255	6.51**	4.35	7.01
		β-glucosidase	4	238	59	1.52	-1.60	20.22
		Viscozyme L	4	747	187	4.76*	10.08	0.99
	Glucose	Celluclast 1.5L	4	6.44	1.61	11.47***	0.92	5.38
		β-glucosidase	4	0.71	0.18	1.27	0.19	31.86
		Viscozyme L	4	0.86	0.22	1.54	0.69	0.91
DCF (0.75% H_2SO_4)	Reducing sugar	Celluclast 1.5L	4	13175	3294	137.3***	-1.15	6.99
		β-glucosidase	4	1849	462	19.28***	-0.18	24.00
		Viscozyme L	4	641	160	6.68**	-0.37	0.89
	Glucose	Celluclast 1.5L	4	1.37	0.34	4.82*	-1.94	7.20
		β-glucosidase	4	0.43	0.11	1.50	-0.66	12.41
		Viscozyme L	4	0.09	0.02	0.31	0.34	1.02
DCF (0.75%NaOH)	Reducingsugar	Celluclast 1.5L	4	27477	6869	33.28***	0.88	6.78
		β-glucosidase	4	3172	793	3.84*	0.67	28.65
		Viscozyme L	4	1560	390	1.89	2.86	2.31
	Glucose	Celluclast 1.5L	4	9.90	2.48	18.18***	0.92	6.05
		β-glucosidase	4	4.73	1.18	8.68**	2.28	30.18
		Viscozyme L	4	0.97	0.24	1.79	0.41	0.90

[a]Degree of freedom, [b]Sum of squares, [c]Mean square

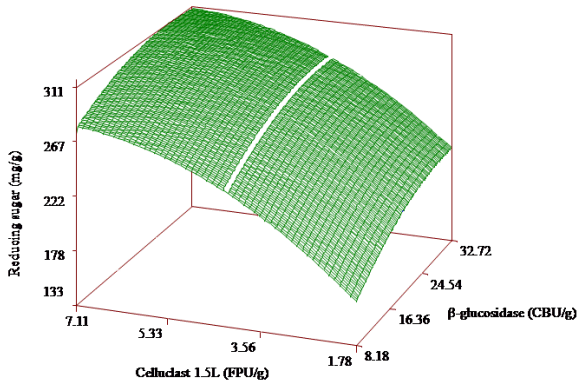

Figure 3: 3D surface plot for reducing sugar yield of alkaline treated destarched corn fiber (DCF) versus Celluclast 1.5 L (FPU/g) and β-glucosidase (CBU/g). Fixed Viscozyme L = 0.82 FBG/g of DCF

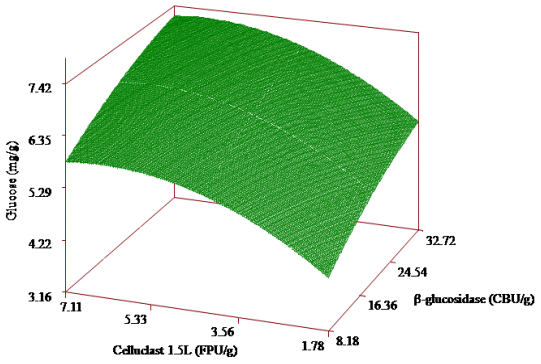

Figure 4: 3D surface plot for glucose yield of alkaline treated destarched corn fiber (DCF) versus Celluclast 1.5 L(FPU/g) and β-glucosidase (CBU/g). Fixed Viscozyme L = 0.82 FBG/g of DCF

The F-test also suggested that the models had a high model *F*-value (F= 17.19; F=12.58) and very low *p*-value (P<0.0030; p<0.0062) (Table 6), indicating that the developed models for alkaline treated DCF were significant and could adequately represent the relationship among the parameters. Moreover, the coefficient of variation (CV) describes the extent to which the data were dispersed.

The CV for reducing sugar and glucose yields of alkaline treated DCF (5.89 and 6.54) was within the acceptable range. Since CV is a measure expressing standard deviation as a percentage of the mean, the small values of CV give better reproducibility. A plots of observed and predicted values indicated an excellent fit (R^2=0.968) for reducing sugar and (R^2= 0.957) for glucose yields of alkaline treated DCF. Therefore, these models

could be used for theoretical prediction of the reducing sugar and glucose yields of alkaline treated DCF.

Analysis of response surfaces

Since the models for reducing sugar and glucose yields of alkaline treated DCF showed insignificant lack of fit, the responses were sufficiently explained by the regression equations. The relationship between independent and dependent variables is illustrated in three dimensional representations of the response surfaces. On the basis of the coded data, since canonical analysis for the glucose yield of alkaline treated DCF demonstrated a saddle point as the stationary point, a ridge analysis was performed to determine the critical levels of the design variables that may produce the maximum glucose yield. The critical values in terms of coded and uncoded variables for reducing sugar and glucose yields are given in Table 7. Figures 3 and 4 represent the response surface plots representing the effect of Celluclast 1.5L and β-glucosidase dosages on reducing sugar and glucose yields of alkaline treated DCF, while keeping Viscozyme L constant. It was observed that as Celluclast 1.5L and β-glucosidase dosage increases, reducing sugar and glucose yield also increases, suggesting a linear relationship between these two variables. Celluclast 1.5L dosage considerably influences the release of reducing sugar and glucose yield at constant Viscozyme L. The release of reducing sugar and glucose is almost dependent on Celluclast 1.5L.

Table 8
Comparison of predicted and observed values of reducing sugar and glucose yields (mg g^{-1}) of alkaline treated destarched corn fiber (DCF)

Pretreatment	Sugar	Eigen value	Stationary point	[a]Predicted value	[b]Observed value
	Reducing				
DCF (0.75%NaOH)		-1.26,-12.22,-23.05	Maximum	316.20	314.93±1.43
	Glucose	0.27,-0.17,-0.56	Saddle	6.90	6.82±1.95

[a]Predicted value using ridge analysis of the response surface quadratic model
[b]Mean ± standard deviation of triplicate determination

As shown in Table 3, at the lowest dosages of Celluclast 1.5L and β-glucosidase, the amount of reducing sugar and glucose yields were 149.71 and 3.71 mg/g, which were increased to 204.60 mg/g and 4.79 mg/g with an increase in Celluclast 1.5L. The maximum reducing sugar and glucose yields, 303.22 and 6.84mg/g, were obtained at the highest dosages of Celluclast 1.5L and β-glucosidase.The curvature nature of the surface plot in Figures 3 and 4 indicates a mutual interaction between Celluclast 1.5L and β-glucosidase.[43]

Based on the values estimated by the regression models, maximum reducing sugar and glucose yields from alkaline treated DCF were predicted at dosages of 6.78 and 6.05 FPU/g of DCF for Celluclast 1.5L, 28.65 and 30.18 CBU/g of DCF for β-glucosidase, and 2.31 and 0.90 FBG/g of DCF for Viscozyme L, respectively. Celluclast 1.5 L had the greatest impact on the reducing sugar and glucose yields of alkaline treated DCF with p = 0.0030 and p = 0.0062, respectively. A previous study on enzymatic saccharification of lignocellulosic substrates used cellulase loadings in the range of 7-33 FPU/g substrates.[44]This is a lower cellulase dosage than those used in most other previous studies. Yoo*et al*.[23] also used the optimum amount of Celluclast 1.5 L, of 6.74 FPU/g cellulose for saccharification of acid treated soy bean hulls.

The addition of β-glucosidase achieved better saccharification by hydrolyzing cellobiose. Cellulase has been supplemented with β-glucosidase in many other studies. Previous studies on enzymatic saccharification of corn fiber used a β-glucosidase (Novozyme 188) loading of 25U/g cellulose[12]and for the saccharification of acid treated soy bean hulls a β-

glucosidase loading of 25.95 CBU/g cellulose was used.[23] Therefore, the optimum dosage of β-glucosidase found in our investigation (28.65 and 30.18 CBU/g of DCF) was comparable with those of previous studies.

The presence of the cell wall degrading enzyme (Viscozyme L) complex did contribute to the efficiency of enzymatic saccharification. The primary mechanism was the hydrolysis of the residual hemicellulose and the increase in cellulase accessibility to the cellulose.[45]Yoo*et al*.[23]reported the use of a dosage of 15.93 FBG/g cellulose ofViscozyme L in the saccharification of acid treated soy bean hulls.

Verification of the model

Verification experiments for the fitted model performed at the predicted enzyme dosages derived from RSM demonstrated that the experimental values were reasonably close to the predicted values, confirming the validity and adequacy of the predicted model. Moreover, the verification experiment also proved that the predicted enzyme dosage for reducing sugar yield could be satisfactorily achieved within the 99% confidence interval of experimental values. The average reducing sugar and glucose yields (314.93 and 6.82 mg/g) are within the predicted value of the model equations (Table 8).

CONCLUSION

This study indicated that Celluclast 1.5L has the main contribution to the reducing sugar and glucose yields of alkaline treated DCF. Alkaline pretreatment significantly decreased the lignin contentand modified the microstructure of destarched corn fiber for efficient enzymatic saccharification. Finally, the high correlation of the model demonstrated that the second-order polynomial model could be used for predicting the maximum fermentable sugar yield of alkaline treated DCF. Enzyme dosages of 6.78 and 6.05 FPU/g of DCF for Celluclast 1.5L, 28.65 and 30.18 CBU/g of DCF for β-glucosidase, and 2.31 and 0.90 FBG/g of DCF for Viscozyme L, respectively,were predicted for achieving maximum reducing sugar and glucose yields of alkaline treated DCF.

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REFERENCES

[1] C. Abbas, K. Beery, E. Dennison and P. Corrington, in “Lignocellulose Biodegradation”, edited by B. C. Saha and K. Hayashi, American Chemical Society, Washington DC, 2004, pp. 84-97.
[2] M. Moniruzzaman, *Appl. Biochem. Biotechnol.*, **59**, 297 (1996).
[3] T. Torget, P. Walter, M. Himmel and K. Grohmann, *Appl. Biochem. Biotechnol.*, **28/29**, 85(1991).
[4] R. Kumar and C. E. Wyman, *Biotechnol. Bioeng.*, **103**, 267 (2009).
[5] R. Kumar and C. E. Wyman, *Biotechnol. Prog.*, **25**, 819 (2009).
[6] Y. Li, R. Ruan, P. L. Chen, Z. Liu, X. Pan, X. Lin *et al*., *Trans. ASAE*, **47**, 825 (2004).
[7] M. Abraham and G.M. Kurup,*Appl.Biochem. Biotechnol*.,**62**, 211(1997).
[8] V. S. Chang and M. T. Holtzapple, *Appl. Biochem. Biotechnol*., **84-86**, 37 (2000).
[9] L. Myat and G.-H. Ryu, *Cellulose Chem. Technol*., **50**, 188 (2016).
[10] R. E. T. Drissen, R. H. W. Mass, J. Tramper and H. H. Beeftink, *Biocatal. Biotransform*., **27**, 35 (2009).
[11] M. Jorge, G. Roberto, P. J. K. Karina, C. E. Oliva, R. Benjamin *et al*., *Int. J. Food Sci. Technol*., **41**, 736 (2006).
[12] A. Altan, K. L. McCarrthy and M. Maskan, *J. Food Eng*., **89**, 32 (2008).
[13] M. Gaspar, G. Kalman and K. Reczey, *Process Biochem*., **42**, 1139 (2007).
[14] B. C. Saha, L. B. Iten, M. A. Cotta and Y. V. Wu, *Process Biochem*., **40**, 3700 (2005).
[15] Z. Wang, D. R. Keshwani, A. P. Redding and J. J. Cheng, *Bioresour. Technol*., **10**, 1016 (2010).
[16] P.J.Wright andA.F.A. Wallis, *Tappi J*., **81**, 130 (1998).
[17] S.G. Douglas, *Food Chem*.,**7**, 145(1981).
[18] H. Cheng, H. Zhan, S. Fu and A. Lucia, *Bioresources*, **11**, 206(2010).
[19] D. Van Eylen, F. van Dongen, M. Kabel and J. de Bont, *Bioresour. Technol*., **102**, 6004 (2011).
[20] H. Jin, C. Zha and G. Lixia, *Carbohyd. Res*., **342**, 858 (2007).
[21] L. Segal and C.M. Conrad, *Am. Dyes Report*, **46**, 642 (1957).
[22] J. D. Gutherie, “The Chemistry of Lint Cotton”, Interscience Publishers Inc., New York, USA, 1955, pp. 27-28.
[23] J. Yoo, S. Alavi, P. Vadlani and V. Amanor-Boadu, *Bioresour. Technol*., **102**, 7590 (2011).
[24] G. C. Miller, *Anal. Chem*., **31**, 428(1959).
[25] G. E. P. Box and N. R. Draper, “Empirical Model-Building and Response Surface”, New York, USA, 1987, pp. 669.

[26] C. N. Hamelinck, G. van Hooijidonk and A. P. C. Faaij, *Biomass Bioeng*., **28**,410 (2005).
[27] N. Moiser, C. Wyman, B. Dale, R. Elander, Y.Y. Lee *et al*., *Bioresour. Technol*., **96**, 686 (2005).
[28] R. P. Chandra, R. Bura, W. E. Mabee, A. Berlin, X. Pan *et al*., Adv. *Biochem. Eng. Bioethanol*., **108**, 93(2007).
[29] R. Kumar and C.E. Wyman, *Biotechnol. Prog*., **25**, 314 (2009).
[30] C. E. Wyman, "Handbook on Bioethanol: Production and Utilization", Taylor and Francis, Washington DC, USA, 1996.
[31] M. G. Jackson, *Anim. Feed Sci. Technol*., **2**, 130 (1977).
[32] R. R. Spencer and D. E. Akin, *J. Anim. Sci*., **51**, 1196 (1980).
[33] L. T. Fan, M. M. Gharpuray and Y. Y. Lee, "Cellulose Hydrolysis", Springer-Verlag Publishers, Germany, 1987, pp. 216.
[34] P. J. Weimer, J. M. Hackney and A. D. French, *Biotechnol. Bioeng*., **48**, 178(1995).
[35] M. Yoshida, Y. Liu, S. Uchida, K. Kawarada, Y. Ukagami *et al*., *Biosci. Biotechnol. Biochem*., **72**, 810 (2008).
[36] X. B. Zhao, L. Wang and D. H. Liu, *J. Chem. Technol. Biotechnol*., **83**, 956(2008).
[37] D. Y. Corredor, X. S. Sun, J. M. Salazar, K. L. Hohn and D. Wang, *J. Biobased Mater. Bio*., **2**, 50 (2008).
[38] S. Lee, Y. Teramoto and T. Endo, *Bioresour. Technol*., **100**, 279(2009).
[39] H. Tarkow and W. C. Feist, in "Cellulases and Their Applications", edited by R. F. Gould, American Chemical Society, Washington, DC, 1969, pp. 197-218.
[40] X. Zhao, L. Zhang and D. Liu, *Bioresour. Technol*., **99**, 3736 (2008).
[41] X. Guan and H. Yao, *Food Chem*., **106**, 351 (2008).
[42] M. Vazquez, R. Delgado and A. J. Castro, *Starch*, **61**, 609 (2009).
[43] X. Yuan, J. Liu, G. Zeng, J. Shi, J. Tong *et al*., *Renew. Energ*., **33**, 1684 (2008).
[44] Y. Sun and J. Cheng, *Bioresour. Technol*., **83**, 11 (2002).
[45] L. T. Fan, Y. Lee and D. H. Beardmore, *Biotechnol. Bioeng*., **22**, 199(1980).