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Effect of hemicellulase, cellulase, xylanase and alkali pretreatment on the saccharification of *Miscanthus sacchariflorus* var. No.1

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The optimum conditions for enzymatic treatment of *Miscanthus sacchariflorus* var. No. 1, a newly developed genotype in Korea, to produce water soluble carbohydrate were investigated using statistical methods. The individual effects of cellulase, hemicellulase and xylanase on water soluble carbohydrate (WSC) production were investigated. Then, the effects of combined addition of enzyme and *M. sacchariflorus* were evaluated and optimized using a Box Benhken design (BBD) and response surface model (RSM). Enzyme treatment was performed in a 45°C water bath for 12 h. The effects of alkali pretreatment conditions on enzymatic saccharification of *M. sacchariflorus* were investigated using the Taguchi design. Hemicellulase was the effective enzyme for WSC yield (mg of produced glucose/g *M. sacchariflorus*). This enzyme then became the fixed component, with cellulase, xylanase and *M. sacchariflorus* applied as variables. The optimum blend of enzymes was determined to be hemicellulase 5.0 U/ml, cellulase 8.0 U/ ml and xylanase 6.0 U/ ml. Incubation time, concentration of NaOH and *M. sacchariflorus* level were used as variables for investigation of alkali pretreatment conditions effects. The pretreatment was performed at 80°C and the optimized enzyme mixture was used. The maximum WSC yield was achieved using a 1 h reaction time, 1% NaOH and 50 g/L of *M. sacchariflorus*. Through a series of experiments, WSC yield was improved from 10 mg glucose/g *M. sacchariflorus* before optimization to 220 mg glucose/g *M. sacchariflorus* after optimization.

Key words: *Miscanthus sacchariflorus* var., enzymatic saccharification, cellulase, hemicellulase, xylanase.

INTRODUCTION

Bioethanol, which is derived from lignocellulosic biomass and not from sugar- or starch-based materials, is considered the most important bio-energy resource (Taherzadeh and Karimi, 2008). Miscanthus is a perennial rhizomatous grass that has been identified as a potentially valuable and environmentally friendly cellulosic biomass for the production of bio-energy by virtue of its highly positive energy balance, with an output to input energy ratio above 10 : 1 (Lewandowski et al., 2000).

Ethanol can be produced from lignocellulosic biomass via saccharification (conversion of cellulosic material to simple sugar) and alcohol fermentation. Saccharification can be divided into physical, chemical and biological

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types of treatment (Jeya et al., 2009; Ma et al., 2009). Of these, enzymatic saccharification is especially favored, given its relative lack of process-related pollution (Gupta et al., 2011)

Lignocellulosic biomass consists of polymeric carbohydrates, mainly cellulose, hemicelluloses, and lignin (Lee et al., 2010). Xylans are the major hemicellulose in grasses (Scheller and Ulvskov, 2010).

Cellulose is a major component of cellulosic material in plants. It is crystalline via β-1,4 glycosidic linkages. Hemicellulose has relative weak crystal structure compared to cellulose and primarily consists of hetero-β-1,4-D-xylan (Poutanen et al., 1986; Nishiyama et al., 2003; Wada et al., 2008).

Degradation of cellulosic biomass can be influenced by its constituents and their structure (Kumar et al., 2009). Elimination of hemicellulose present in cellulosic biomass can improve the digestibility of cellulose (Allen et al., 2001; Kabel et al., 2007). Xylan is an inhibition factor for cellulose degradation and the removal of its oligomer form, xylan, can improve the cellulose degradation (Qing et al., 2010). Therefore, the synergistic action of cellulolytic hydrolysis by various enzymes produced by microorganisms is essential for the achievement of high saccharification yield (Woodward, 1991; Suwannarangsee et al., 2012).

In the present study, enzymatic saccharification of *M. sacchariflorus* var No. 1, a newly developed genotype in Korea (Moon et al., 2010) was studied for the first time. Particularly, the synergistic mixture of cellulase, hemicellulase and xylanase was optimized using statistical techniques and the effects of alkali pretreatment conditions were clarified.

METHODOLOGY

Plant material

M. sacchariflorus var. No. 1 (hereafter referred to as Miscanthus) was obtained at late maturity stage (full bloom) from the Bioenergy Crop Research Center, National Institute of Crop Science, Muan, Korea. Miscanthus was dried at 60°C for 48 h and was ground to sizes <1 mm in length using a NF10.1 laboratory grinder (ICA, Staufen, Germany).

Enzymes and chemicals

Enzyme solutions and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. The used cellulase (EC 3.2.1.4) and hemicellulase (CAS 9025-56-3, mixture of xylanase, mananase and other glycolytic enzymes) were produced from *Aspergillus* sp. and *A. niger*, respectively. The used xylanase (EC 3.2.1.8) was produced by *Thermomyces lanuginosus*. The enzyme solutions of cellulase, hemicellulase and xylanase were diluted to 1,000, 3,000 and 2,500 U/g, respectively before being used in this experiment. One unit (U) of each enzyme is defined as the release of 1 µmol of glucose at 37°C and pH 5.0 for cellulase, at 40°C and pH 5.0 for hemicellulase and at 30°C and pH 4.0 for xylanase. Hemicellulase and xylanase were diluted with 10 volumes of 37 mM acetic acid buffer (pH 4.8) before use.

Reaction conditions

Miscanthus was suspended in 37 mM acetic acid buffer, pH 4.8, and mixed with the particular enzyme solution(s). The mixture was incubated in a water bath operating at 45°C for 12 h with agitation at 150 rpm.

Determination of water soluble carbohydrate

The water soluble carbohydrate (WSC) content was determined by measuring reducing sugar concentration (Arrizon et al., 2010). After enzyme reaction, the mixture was centrifuged (10,000 rpm, 5 min) and the supernatant was used for WSC determination. WSC was analyzed according to spectrophotometric detection of reducing sugar with a solution of 3,5-dinitrosalicyclic acid. Glucose was used as standard for the regression. WSC production rate was calculated as the production of mg of glucose per utilized g of Miscanthus powder.

Effect of enzymes on WSC production yield

To find the most effective enzyme in terms of WSC yield, each enzyme was individually added to reaction mixtures at 0.1, 1.0 and 10.0 U/mL, along with 10 g/L Miscanthus. The enzyme and its concentration that showed the greatest yield displayed were fixed and the remaining 2 enzymes and Miscanthus were used as variables. 3 levels of each variable were configured according to the Box Behnken design (Box and Behnken, 1960) and 30 runs with duplicated experiment groups were carried out (Table 1). After enzymatic reaction, WSC yields from each trial were determined and used as a response from the BBD system. The behavior of system was calculated using the following response surface model (RSM):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_i X_i^2 + \sum \beta_{ij} X_i X_j$$

where Y means predicted response (WSC yield), β_0 is a constant, $\beta_i$and $\beta_j$are regression coefficients and X_i and X_j are independent variables ($i \neq j$).

Effects of alkali pretreatment conditions

Effects of alkali pretreatment conditions on WSC production in optimized enzymatic saccharification system were investigated using a Taguchi experimental design (Periasamy and Palvannan, 2010) Reaction time (h), concentration of NaOH (%) and content of Miscanthus (g/L) were used as variables. The variables were orthogonally arranged and a total of 9 trials were produced by the experimental design. After pretreatment according to the experimental design, the pH of the mixture was adjusted to 4.8 using acetic acid and enzymatic hydrolysis with the previously optimized enzyme blend was performed. The WSC yield from each trial was used as a response and the signal-to-noise (SN) ratio was calculated with the response using an equation for larger-the-best characteristics:

$$SN = -10\log\left(\frac{1}{r}\sum_{i=1}^{r}\frac{1}{R_i^2}\right)$$

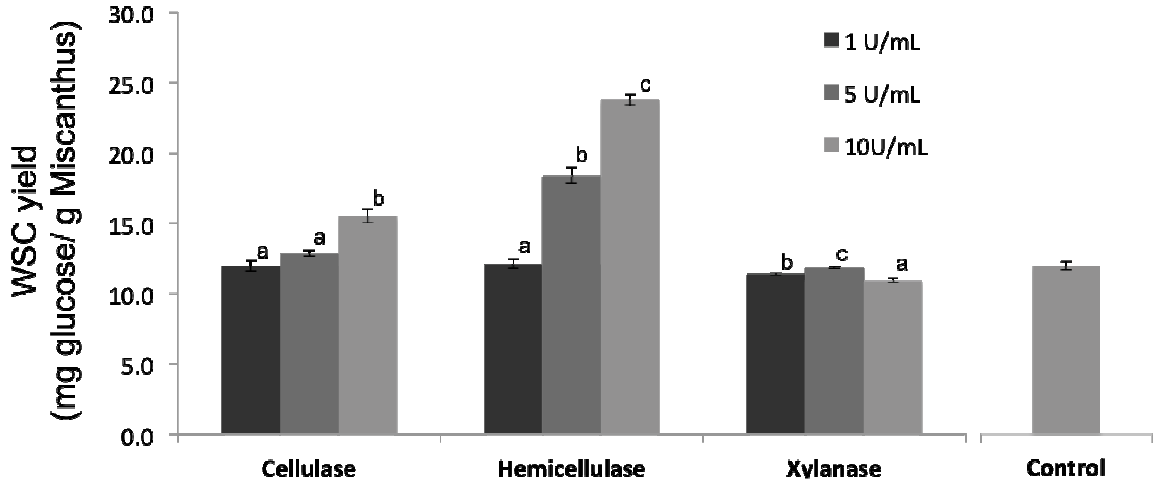

Figure 1. Effects of cellulase, hemicellulase and xylanase concentration on water soluble carbohydrate (WSC) yield from Miscanthus. Enzyme reaction was performed in 37 mM acetic acid buffer (pH 4.8) with 50 g/L of Miscanthus at 45°C for 12 h.

where r and R_i mean total number of trials and responses from the i^{th} trial, respectively.

Statistical analysis

All experiments were performed in triplicate. Multiple comparisons of WSC yields by different concentrations of enzymes were performed using a general linear model and means were separated using the Duncan's multiple range test. The SPSS software (version 20.0.0) was used. Construction of BBD and Taguchi design, normal distribution test of responses and analysis of variance for the responses from Taguchi design were performed using MINITAB® version 14.0 (Minitab, PA, USA) as a statistical software.

RESULTS

Effects of individual enzymes

The WSC yields from Miscanthus resulting from the individual application of cellulase, hemicellulase and xylanase are summarized in Figure 1. WSC yields in the presence of cellulase and hemicellulase, but not xylanase, were increased according to elevated enzyme activities. The highest WSC yield was found using 10 U/mL hemicellulase ($p < 0.05$). Hemicellulase was selected as the fixed variable for the next experiment and it was fixed to half of maximum activity (5 U/ml) to bring other enzymes used as variable into relief.

Optimization of enzyme mixture

The amounts of Miscanthus were varied from 50 to 200 g/L and the activities of cellulase and xylanase were varied from 8 to 2 U/ml. In all trials, hemicellulase was added at 5.0 U/mL. Various WSC yields in each trial were varied from 25.3 to 44.0 g glucose/g Miscanthus (Table 1). Analysis of variance of the responses was determined (Table 2). Probabilities of regression, linear and square effects were significant ($P < 0.001$) whereas interaction among variables was not significant ($P > 0.05$). Lack-of-fit was also not significant ($P > 0.05$). The responses were fitted to response surface model and the following regression was achieved:

$$\text{WSC yield} = 40.97 - 0.27X_1 + 1.53X_2 + 1.88X_3 + 8.0\text{E-}04X_1^2 - 0.12X_2^2 - 0.17X_3^2 - 7.0\text{E-}04X_1X_2 - 1.4\text{E-}04X_1X_3 + 0.06X_2X_3$$

where X_1, X_2 and X_3 are variables of Miscanthus, cellulase and xylanase, respectively. The determination coefficient of regression was 93.4%.

Standardized residual errors (SREs) from the comparison of the actual WSC yields and predicted yields from achieved regression are summarized in Table 1. All SREs were under ± 2.0, except that from trial 17 (2.44). The main effects of the variables are depicted in Figure 2. WSC yield was decreased along with increased levels of Miscanthus (Figure 2A). Similar patterns of main effects were found in both of cellulase and xylanase. WSC yield was increased from 2 to 5 U/ml of enzyme and was not increased from 5 to 8 U/mL (Figure 2B and C). The interactions between cellulase and xylanase at different levels of Miscanthus are summarized in Figure 3. The highest yields were found between 7 U/mL of cellulase and 6 U/ml of xylanase, regardless of the levels of Miscanthus. Optimum condition for enzymatic treatment of Miscanthus was calculated via optimization using RSM as 50 g/L of Miscanthus, 8.0 U/ml of cellulase, 6.0 U/ml of xylanse and 5.0 U/ml of hemicellulase. The predicted yield of WSC was 41.44 mg

Table 1. Configuration of 30 trials according to the Box Behnken design and their observed and predicted responses.

Runs	Variables[1]						Responses		
	Coded values			Actual values			Actural yield[2]	Predicted	SRE[3]
	X_1	X_2	X_3	X_1 (g/L)	X_2 (U/ml)	X_3 (U/ml)			
1	-1	-1	0	50	2	5	35.6	37.4	-1.48
2	1	-1	0	200	2	5	25.3	26.1	-0.76
3	-1	1	0	50	8	5	39.4	41.0	-1.26
4	1	1	0	200	8	5	29.1	29.1	-0.02
5	-1	0	-1	50	5	2	36.6	37.6	-0.79
6	1	0	-1	200	5	2	26.5	26.6	-0.11
7	-1	0	1	50	5	8	37.6	39.9	-1.86
8	1	0	1	200	5	8	27.4	27.7	-0.27
9	0	-1	-1	100	2	2	27.2	27.7	-0.43
10	0	1	-1	100	8	2	28.7	29.9	-1.04
11	0	-1	1	100	2	8	29.3	28.5	0.66
12	0	1	1	100	8	8	33.3	32.9	0.30
13	0	0	0	100	5	5	31.8	32.4	-0.44
14	0	0	0	100	5	5	31.7	32.4	-0.52
15	0	0	0	100	5	5	31.8	32.4	-0.44
16[4]	-1	-1	0	50	2	5	36.8	37.4	-0.52
17	1	-1	0	200	2	5	26.6	26.1	0.35
18	-1	1	0	50	8	5	44.0	41.0	2.44
19	1	1	0	200	8	5	30.6	29.1	1.31
20	-1	0	-1	50	5	2	40.0	37.6	1.95
21	1	0	-1	200	5	2	26.7	26.6	0.07
22	-1	0	1	50	5	8	41.8	39.9	1.52
23	1	0	1	200	5	8	27.0	27.7	-0.57
24	0	-1	-1	100	2	2	29.0	27.7	1.08
25	0	1	-1	100	8	2	29.0	29.9	-0.78
26	0	-1	1	100	2	8	29.9	28.5	1.16
27	0	1	1	100	8	8	31.8	32.9	-0.95
28	0	0	0	100	5	5	32.8	32.4	0.28
29	0	0	0	100	5	5	33.6	32.4	0.85
30	0	0	0	100	5	5	32.8	32.4	0.28

[1]Variables of X_1, X_2 and X_3 are Miscanthus, cellulase and xylanase, respectively. [2]Yield is mg glucose produced from g Miscanthus. [3]SRE means standardized residual error value. [4]Run 16 to 30 are replications of experiment.

glucose/g Miscanthus.

Effect of alkali pretreatment conditions

Alkali pretreatment was applied to increase WSC yield of enzymatic saccharification of Miscanthus. For variables, reaction time was varied from 1 to 3 h, the concentration of NaOH was varied from 0.5 to 2.0% and Miscanthus was varied from 50 to 200 g/L. The optimum enzyme mixture found in BBD experiment was used for following enzymatic saccharification. 9 trials were conducted (Table 3). The observed responses were used to calculate the SN ratio (Table 3). The probabilities of the main effects of the variables were calculated via ANOVA (Table 4). Variables of NaOH and Miscanthus were significant ($P < 0.05$). Main effects obtained using average SN ratios are plotted in Figure 4. The highest SN ratios were found at 2 h of incubation time (Figure 4A), 1% of NaOH (Figure 4B) and 50 g/L of Miscanthus (Figure 4C). Finally, the enzymatic saccharification of Miscanthus was performed using the optimum condition. First, 50 g/L of Miscanthus was pretreated using 1% NaOH in an 80°C water bath for 2 h. The pH of the mixture was adjusted to 4.8 using acetic acid and the enzymatic treatment was performed with 5.0 U/ml of hemicellulase, 8.0 U/ml of cellulase and 6.0 U/ml of xylanase in a 45°C water bath for 12 h. The WSC yield from this condition was 220 ± 5.3 mg glucose/g Miscanthus.

Table 2. Analysis of variance[1] for the response[2] from the Box Benhken design experiment.

Sources	DF	SS	MS	F value	P value
Regression	9	660.842	73.427	31.70	<0.001
Linear	3	506.289	64.006	27.63	<0.001
Square	3	150.072	50.024	21.59	<0.001
Interaction	3	3.482	1.161	0.50	0.686
Residual error	17	46.329	2.316		
Lack-of-fit	3	12.363	4.121	2.06	0.143
Pure error	14	33.966	1.998		
Total	26	707.172			

[1]DF, degree of freedom; SS, sum of square; MS, mean of square.

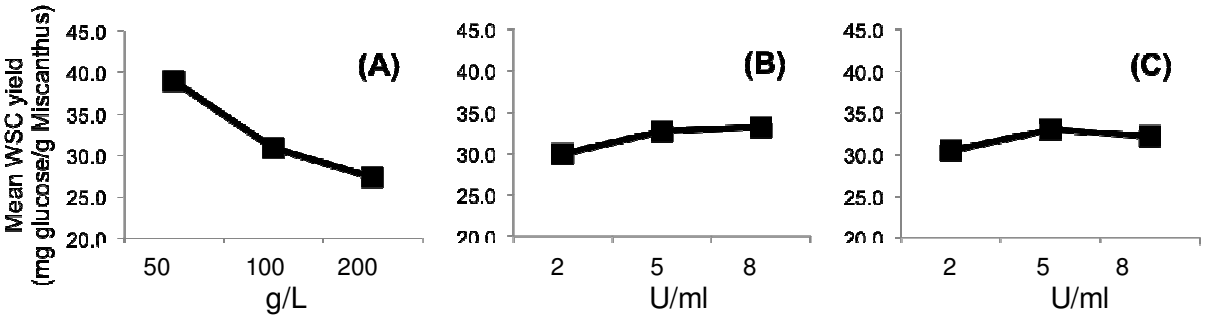

Figure 2. Main effect of the variables on WSC yield. (A), (B) and (C) are levels of Miscanthus in reaction mixture, activity of cellulase and activity of xylanase, respectively.

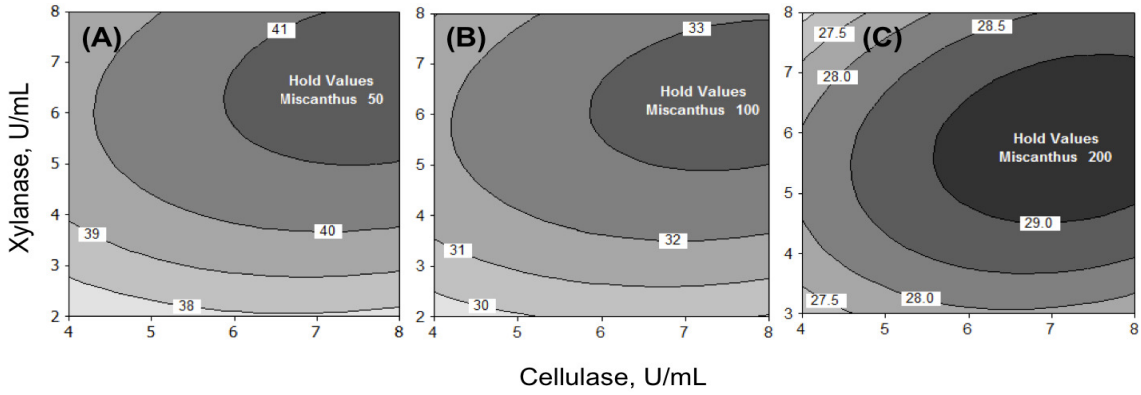

Figure 3. Contour plot for the interaction between cellulase and xylanase on WSC yield under various levels of Miscanthus in reaction mixture. (A), (B) and (C) are the amount of used Miscanthus as 50 g/L, 100 g/L and 200 g/L, respectively. The plots were calculated via achieved quadratic regression model.

DISCUSSION

Conversion of lignocellulosic biomass to simple sugar, which is a fuel for ethanol production, is very dependent on the characteristics of feedstocks and the treatment technology. Enzymatic saccharification of cellulosic biomass can be successfully accomplished by a heterogeneous hydrolysis via the synergistic action of various enzymes (Xiao et al., 2004). However, the hydrolytic enzyme can be restricted by an impeded or coated carbohydrate across cellulose (Qing et al., 2010). The enzymatic hydrolysis of cellulose can be facilitated by removal of hemicellulose and xylose (Allen et al., 2001; Kabel et al., 2007). Addition of hemicellulase and xylanase can enhance cellulase-catalyzed saccharification (Selig et al., 2008). These studies support the existence

Table 3. Taguchi design for the investigation of the effects of alkali pretreatment conditions on WSC production from Miscanthus.

Runs	Coded			Actual values			Responses, WSC yield, mg glucose/g Miscanthus			
	A	B	C	A (h)	B (%)	C (g/L)	R1	R2	R3	SN ratio[1]
1	1	1	1	1	0.5	50	140.40	143.16	130.95	42.79
2	1	2	2	1	1.0	100	120.30	178.89	226.15	43.98
3	1	3	3	1	2.0	200	3.89	13.72	10.47	15.71
4	2	1	2	2	0.5	100	83.68	124.96	155.59	40.82
5	2	2	3	2	1.0	200	45.97	118.54	174.11	37.16
6	2	3	1	2	2.0	50	189.62	186.32	181.07	45.37
7	3	1	3	3	0.5	200	30.22	76.35	107.71	33.46
8	3	2	1	3	1.0	50	196.13	188.61	175.51	45.40
9	3	3	2	3	2.0	100	29.07	45.53	43.02	31.34

[1].Signal and noise ratio was calculated by lager the best characteristic. Used enzyme blend was 5 U/mL of cellulose, 1.5 U/ml of hemicellulase and 1.5 U/mL of xylanase. Alkali pretreatment was performed at 80°C

Table 4. Analysis of variance[1] for the signal-to-noise ratio from the Taguchi experiment.

Source	DF	SS	MS	F value	P value
Reaction time	2	8377	4189	2.67	0.094
NaOH	2	29288	14644	9.32	0.001
Miscanthus	2	50402	25201	16.04	<0.001
Error	20	31418	1571		
Total	26	119486			

[1]DF, degree of freedom; SS, sum of square; MS, mean of square. R^2=83.87%.

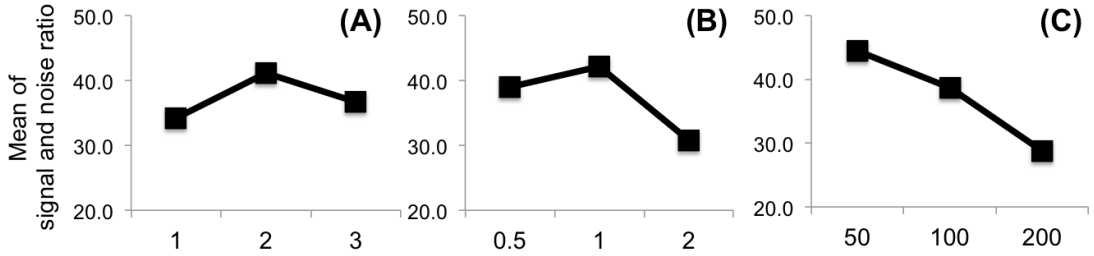

Figure 4. Mean of signal-to-noise ratio from each level of variables for alkali pretreatment. (A), (B) and (C) are reaction time (h), concentration of NaOH (%) and content of Miscanthus (g/L), respectively.

or importance of synergistic interaction among various cellulolytic enzymes on the efficiency of saccharification. In the study of Qing et al., (2010), xylanooligomer reduced the glucose yield during the enzymatic hydrolysis of cellulose by up to 82%. The authors suggested that hemicellulose removal should be prior to enzymatic saccharification for yield enhancement. In the present study, the maximum WSC yield was found between 5 to 7 U/mL of xylanase and 6 to 8 U/mL of cellulase.

For successful saccharification, physico-chemical pretreatment is considered to be a promising process because it can reduce the crystallinity of cellulosic biomass and ensure sufficient surface for the binding of cellulase (Guo et al., 2009). Although the degree of sugar yield enhancement in enzymatic saccharification can differ according to types of pretreatments, it is commonly accepted that the pretreatment can ensure more than 60% enhancement (Jin and Chen, 2006; Hideno et al., 2009).

There are various strategies for the pretreatment of cellulosic biomass. The pretreatments using dilute acids have been reported to enhance enzymatic saccharification by removal of hemicellulosic fraction and can result in subsequent conversion of hemicellulose to fermentable sugars (Schell et al., 2003; Guo et al., 2009). Alkali pretreatments can improve saccharification by removal of lignin and uronic substituents responsible for

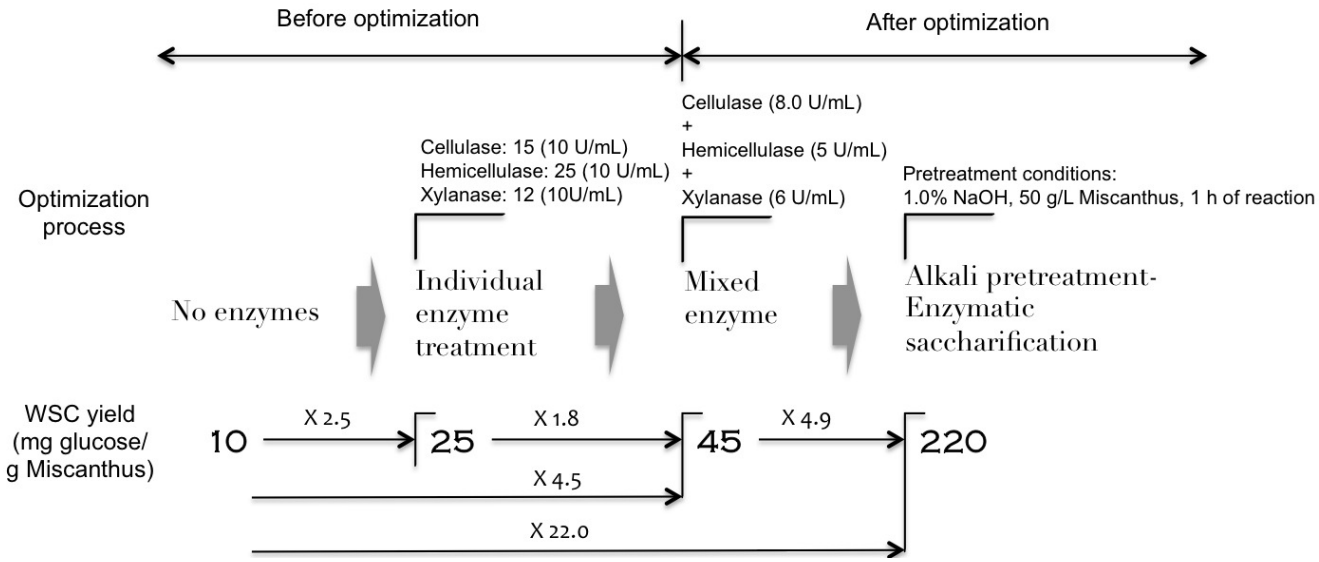

Figure 5. Schematic flow for the enhancement of water soluble carbohydrate production via optimization.

inhibition of cellulase binding and by cleaving the lignin bond linkage with the cell wall hemicellulose and cellulose constituents (Gupta et al., 2011). For other pretreatments, sodium chlorite, chlorine dioxide, microwave, heat and milling treatment have been applied to enhance enzymatic saccharification (Hideno et al., 2009; Jeya et al., 2009; Ma et al., 2009; Binod et al., 2012).

Conclusion

An enzyme mixture of hemicellulase, cellulase and xylanase and alkali pretreatment condition for the saccharification of Miscanthus was optimized. Hemicellulase proved to be the most efficient enzyme for WSC yield and a synergistic effect was evident between cellulase and xylanase. The optimum enzyme mixture consisted of 5.0 U/ml of hemicellulase, 8.0 U/ml of cellulase and 6.0 U/ml xylanase. With a 1-h reaction time, 1.0% NaOH and 50 g/L Miscanthus were optimum for enzymatic saccharification of Miscanthus; the WSC yield was increased by 22 times compared to the yield prior to optimization. Therefore, the present study providing primary information for the enzymatic saccharification of newly developed *M. sacchariflorus* var. No 1.

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REFERENCES

Allen SG, Schulaman D, Lichwa J, Antal MJJ (2001). A comparison of aqueous and dilute-acid single-temperature pretreatment of yellow poplar sawdust. Ind. Eng. Chem. Res. 40:2352-2361.

Arrizon J, Morel S, Gschaedler A, Monsan P (2010). Comparison of the water-soluble carbohydrate composition and fructan structures of *Agave tequilana* plants of different ages. Food Chem. 122:123-130.

Binod P, Satyanagalakshmi K, Sindhu R, Janu KU, Sukumaran RK, Pandey A (2012). Short duration microwave assisted pretreatment enhances the enzymatic saccharification and fermentable sugar yield from sugarcane bagasse. Renew. Energy 37:109-116.

Box GEP, Behnken DW (1960). Some new three level designs for the study of quantitative variables. Technometrics 2:455-475.

Guo GL, Hsu DC, Chen WH, Chen WH, Hwang WS (2009). Characterization of enzymatic saccharification for acid-pretreated lignocellulosic materials with different lignin composition. Enzyme Microb. Technol. 45:80-87.

Gupta R, Khasa YP, Kuhad RC (2011). Evaluation of pretreatment methods in improving the enzymatic saccharification of cellulosic materials. Carbohydr. Polym. 84:1103-1109.

Hideno A, Inoue H, Tsukahara K, Fujimoto S, Minowa T, Inoue S, Endo T, Sawayama S (2009). Wet disk milling pretreatment without sulfuric acid for enzymatic hydrolysis of rice straw. Bioresour. Technol. 100:2706-2711.

Jeya M, Zhang YW, Kim IW, Lee JK (2009). Enhanced saccharification of alkali-treated rice straw by cellulase from *Trametes hirsuta* and statistical optimization of hydrolysis conditions by RSM. Bioresour. Technol. 100:5155-5161.

Jin S, Chen H (2006). Structural properties and enzymatic hydrolysis of rice straw. Process Biochem. 41:1261-1264.

Kabel MA, Bos G, Zeevalking J, Voragen AGJ, Schols HA (2007). Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresour. Technol. 98:2034-2042.

Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind. Eng. Chem. Res. 48:3713-3729.

Lee SB, Jung SK, Lee JD (2010). Production of rice straw based cellulosic ethanol using acidic saccharification. Appl. Chem. Eng., 21:349-352.

Lewandowski I, Clifton-Brown JC, Scurlock JMO, Huisman W (2000). Miscanthus: European experience with a novel energy crop. Biomass

Bioenergy 19:209-227.
Ma H, Liu WW, Chen X, Wu YJ, Yu ZL (2009). Enhanced enzymatic saccharification of rice straw by microwave pretreatment. Bioresour. Technol. 100:1279-1284.
Moon YH, Koo BC, Choi YH, Ahn SH, Bark ST, Cha YL, An GH, Kim JK, Suh SJ (2010). Development of "Miscanthus" the promising bioenergy crop. Kor. J. Weed Sci. 30:330-339.
Nishiyama Y, Sugiyama J, Chanzy H, Langan P (2003). Crystal structure and hydrogen bonding system in cellulose Iα from synchrotron X-ray and neutron fiber diffraction. J. Am. Chem. Soc., 125:14300-14306.
Periasamy R, Palvannan T (2010). Optimization of laccase production by *Pleurotus ostreatus* IMI 395545 using the Taguchi DOE methodology. J. Basic Microbiol. 50:1-9.
Poutanen K, Puls J, Linko M (1986). The hydrolysis of steamed birchwood hemicellulose by enzymes produced by. Appl. Microbiol. Biotechol. 487-490.
Qing Q, Yang B, Wyman CE (2010). Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. Bioresour. Technol. 101:9624-9630.
Schell DJ, Farmer J, Newman M, McMillan JD (2003). Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor: Investigation of yields, kinetics, and enzymatic digestibilities of solids. Appl. Biochem. Biotechnol. 105-108:69-85.
Scheller HV, Ulvskov P (2010). Hemicelluloses. Annu. Rev. Plant Biol. 61:263-289.
Selig MJ, Knoshaug EP, Adney WS, Himmel ME, Decker SR (2008). Synergistic enhancement of cellobiohydrolase performance on pretreated corn stover by addition of xylanase and esterase activities. Bioresour. Technol. 99:4997-5005.
Suwannarangsee S, Bunterngsook B, Arnthong J, Paemanee A, Thamchaipenet A, Eurwilaichitr L, Laosiripojana N, Champreda V (2012). Optimisation of synergistic biomass-degrading enzyme systems for efficient rice straw hydrolysis using an experimental mixture design. Bioresour. Technol. 119:252-261.
Taherzadeh MJ, Karimi K (2008). Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. Int. J. Mol. Sci., 9:1621-1651.
Wada M, Nishiyama Y, Chanzy H, Forsyth T, Langan P (2008). The structure of celluloses. ICDD, 51:138-144.
Woodward J (1991). Synergism in cellulase systems. Bioresour. Technol. 36:67-75.
Xiao Z, Zhang X, Gregg DJ, Saddler JN (2004). Effects of sugar inhibition on cellulases and beta-glucosidase during enzymatic hydrolysis of softwood substrates. Appl. Biochem. Biotechnol. 113-116:1115-1126.