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Improvement of cassava stem hydrolysis by two-stage chemical pretreatment for high yield cellulosic ethanol production

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Abstract−We used sodium chlorite followed by sodium hydroxide as a two-stage pretreatment of cassava stem for removal of lignin and hemicellulose to obtain a substrate with high cellulose content prior to hydrolysis. Response surface methodology was applied to determine the optimum hydrolysis conditions of two-stage pretreated cassava stem. After pretreatment, the cellulose content of cassava stem increased from 42.10% to 86.45%, concomitant with decreases in lignin (87.59%) and hemicellulose (78.18%) content. Acid hydrolysis of two-stage pretreated cassava stem under optimum conditions allowed obtaining a hydrolyzate rich in reducing sugar, with a yields up to 67.37%. Conversely, inhibitors were detected at very low concentrations. The fermentation of the hydrolyzate resulted in an ethanol yield of 22.58 g/100 g substrate corresponding to a theoretical ethanol yield of 84.41%. The results demonstrate that two-stage pretreatment is effective for improving cellulose hydrolyzability, resulting in high fermentable sugar and low fermentation inhibitor concentrations.

Keywords: Cassava Stem, Two-stage Pretreatment, Response Surface Methodology, Reducing Sugar, Cellulosic Ethanol

INTRODUCTION

Lignocellulosic biomass from forestry, agricultural and agro-industrial residues is an attractive raw material for bioethanol production due to its availability in enormous quantities at low cost, its richness in lignocellulose and its lack of competition with food crops [1].

Thailand is the world's third largest producer and exporter of cassava, following the lead by Nigeria and Brazil. Approximately 20 to 25 million tons of cassava roots are harvested each year from over 1 million hectares (2.50 million acres) of planting area in 48 provinces of the country, 75% of which are processed for export [2]. After harvesting of cassava roots, farm owners usually keep good stems (cuttings) for cultivation in the following year. The abundance of stems causes lignocellulosic biomass residues in the field and the owners will get rid of them by burning or plowing them back into the earth. It has been estimated that the quantity of destroyed stems is 4 million tons per year [3]. Thus, a large quantity of stem residues, rich in lignocellulose, low-cost feed stock is still currently discarded as waste [4]. Fully utilizing cassava stem residues to produce ethanol biofuel would be a highly attractive option.

However, the major problem of bioethanol production from lignocellulosic biomass (cellulosic ethanol) is the low conversion of these materials into fermentable sugars, which is the result of a hemicellulose and lignin matrix affecting the hydrolyzability of cellulose [5]. Pretreatment is essential to disrupt the rigid structure of lignocellulosic biomass and remove non-cellulosic components for increasing the effectiveness of acid or enzymatic hydrolysis of cellulose.

A two-stage chemical pretreatment method appears to be the best approach to both obtain more purified cellulose content and to enhance hydrolysis [6]. Several two-stage chemical pretreatments have been reported, such as a two-stage chemical pretreatment of dilute H_2SO_4 followed by NaOH pretreatment [6], NH_4OH followed by H_3PO_4 pretreatment [7], and NaClO followed by NaOH [8].

Hence, our aim was to improve acid hydrolysis of cassava stem using $NaClO_2$ followed by NaOH as a two-stage chemical pretreatment to obtain the hydrolyzate containing highly fermentable sugar (reducing sugar) and low inhibitor compound concentrations, and to achieve cellulosic ethanol at a high yield in the subsequent fermentation stage. A central composite design (CCD) based on response surface methodology (RSM) was also applied to design an experiment and optimize the conditions of dilute H_2SO_4 hydrolysis of two-stage pretreated cassava stem in order to save money and energy. The concentration of H_2SO_4, ratio of acid volume to sample and hydrolysis time were considered as critical parameters. A regression model was developed for reducing sugar yield and its adequacy was investigated using the Design-Expert software.

EXPERIMENTAL

1. Raw Material

Cassava stems (variety Huay Bong 60) were collected from a cassava planting area in Udon Thani Province, Thailand, after harvest of cassava roots in the summer of 2013. The raw materials were thoroughly washed with tap water, chopped into small pieces and

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dried under sunlight. They were then crushed into powder and sieved to select the particle size of 500 μm (35 mesh). Ground biomass (7.43% moisture content) was stored in sealed containers, in a desiccator until further use.

2. Pretreatment Processes

Two-stage pretreatment was performed for the removal of lignin and hemicellulose to obtain cassava stem with high cellulose content.

2-1. First Stage: Acid-chlorite Pretreatment

Cassava stem was delignified according to a modification of the method outlined by Kumar et al. [9]. Briefly, the dried cassava stem powder was placed in an Erlenmeyer flask and allowed to soak in tap water (16 mL/g biomass). Afterward, sodium chlorite ($NaClO_2$) (1.5 g/g biomass) was added and acetic acid (CH_3COOH) (5.0 μL/g biomass) was slowly incorporated under stirring. With the top of the neck flask covered with aluminum foil, the mixture was heated to 70 °C and the same dose of $NaClO_2$ and CH_3COOH was added to the reaction every 1 h for 3 h. After the end of reaction, the solid residue (white solid) was washed thoroughly with tap water until neutral pH and placed in a hot-air oven at 105 °C until dry. Then, the oven-dried sample was stored for further sodium hydroxide pretreatment in the next stage.

2-2. Second Stage: NaOH Pretreatment

The acid chlorite delignified cassava stems were pretreated to remove hemicellulose by soaking in 0.25 M NaOH (20% w/v) for 24 h and then heated at 80 °C for 1 h in a water bath (modified from Zhang and Cai [10]). The solids were separated by filtration and thoroughly washed with tap water to neutral pH, dried in a hot-air oven and subsequently used as a substrate for cellulosic ethanol production.

3. Biomass Composition Analysis

The chemical composition (hemicellulose, cellulose and lignin) of all cassava stem samples was analyzed in duplicate using the procedure described by Goering and van Soest [11]. Ash content in the samples was determined according to the National Renewable Energy Laboratory (NREL) [12].

4. Dilute H_2SO_4 Hydrolysis

Dilute H_2SO_4 hydrolysis was carried out in a screw cap reagent bottle containing 100 g of dry two-stage pretreated cassava stem. The reaction was performed in an autoclave (All American Pressure Sterilizer, USA) at 121 °C, 15 psi. The H_2SO_4 concentration, ratio of acid volume to sample and hydrolysis time were in the range of 0.10-0.30 M, 10-20 mL/g and 60-120 min, respectively. After hydrolysis, the hydrolyzate was centrifuged at 5,000 rpm for 10 min. Then, the pH of the supernatant was adjusted to 5.50 using 2 M NaOH, and the resulting precipitate was removed by re-centrifugation. The final supernatant of hydrolyzate obtained from the various conditions was estimated for total reducing sugars yield. Upon completion the hydrolysis under optimum conditions, the hydrolyzate was characterized and quantitatively analyzed for each sugar monomers (glucose, xylose and arabinose) and inhibitor compounds (furfural, 5-hydroxymethylfurfural, acetic acid and phenolic compounds).

5. RSM Optimization of the Hydrolysis Conditions

5-1. Experimental Design

CCD was applied for optimization of the acid hydrolysis conditions of two-stage pretreated cassava stem. The acid concentration (A), ratio of acid volume to sample (B) and hydrolysis time (C) were chosen as the independent variables. The hydrolysis experiments were designed to optimize the acid hydrolysis using the Design-Expert 6.0.10 software package (Stat-Ease Inc., Minneapolis, MN. USA). The total number of experiment points could be calculated using the following equation:

$$N=2^K+2K+C_o \quad (1)$$

where N is the number of experiments run, K is the number of variables and C_o is the number of repetitions of the experiments at the center point.

For CCD with three variables (K=3) and four center points (C_o=4), a total of 18 runs of experiments ($N=2^3+2(3)+4=18$) were employed for acid hydrolysis. For statistical calculation, the variables were coded according to Eq. (2):

$$X_i=\frac{(A_i-A_0)}{\Delta A_i} \quad (2)$$

where X_i, A_i are the coded and actual values of the independent variable, respectively. A_0 is the actual value of the independent variable at the center point and ΔA_i is the step changes of A_i.

The ranges and levels of the independent variables studied are shown in Table 1. A second- order response surface model for predicting the optimal point used was

$$Y_i=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{12}AB+\beta_{13}AC+\beta_{23}BC \quad (3)$$

where Y_i is the predicted response (total reducing sugar yield, g/100 g substrate), β_0 is the intercept coefficient, β_1, β_2, β_3 are the linear coefficients, β_{11}, β_{22}, β_{33} are the quadratic coefficients, β_{12}, β_{13}, β_{23} are the cross-product coefficients and A, B, C are the coded levels of the independent variables investigated.

5-2. Model Fitting and Statistical Analysis

All hydrolysis experiments were repeated three times and results expressed as mean values. The results obtained from CCD were used to determine the regression coefficients of the second-order

Table 1. Variables and experimental design levels to study the hydrolysis of two-stage pretreated cassava stem

Variable	Symbol	Coded levels				
		$-\alpha$	−1	0	+1	$+\alpha$
H_2SO_4 concentration (M)	A	0.03	0.10	0.20	0.30	0.37
Ratio of acid volume to sample (mL/g)	B	6.59	10	15	20	23.40
Hydrolysis time (min)	C	40	60	90	120	141

α=1.68

response surface model (quadratic regression model). The analysis of variance (ANOVA) evaluated the goodness of the fit of the quadratic regression model equation. Three-dimensional (3D) surface plot and corresponding 2D contour plots were drawn to illustrate the effects of the selected independent variables on the responses (total reducing sugar yield). The optimum levels for the variables were obtained by solving the regression equation using Design-Expert® 6.0.10.

6. Analytical Methods

Total reducing sugar yield was estimated by the 3,5-dinitrosalicylic acid (DNS) colorimetric method [13]. The yield of total reducing sugar in the form of g/100 g substrate was calculated according to Eq. (4):

$$\text{Total reducing sugar yield (g/100 g substrate)} = \frac{Rc \times V_1 \times 100\ g}{1000\ mL \times M_1} \quad (4)$$

where Rc is the concentration of total reducing sugar (g/L), V_1 is the volume of H_2SO_4 solution added (mL) and M_1 is the weight of two-stage pretreated sample added (g).

Sugar monomers and some inhibitors (furfural, 5-hydroxymethylfurfural and acetic acid) in the hydrolyzate were characterized and quantitatively analyzed by high performance liquid chromatography (HPLC) with a refractive index detector (Waters 2414, USA) and an Aminex HPX-87H column (300 mm×7.8 mm i.d.) (Bio-Rad, Hercules, CA, USA) under the following conditions: column temperature at 65 °C, mobile phase 0.005 M H_2SO_4 and flow rate 0.60 mL/min. The injection volume was 20 μL. Total phenolic compounds (inhibitor) were also determined according to the Folin-Ciocalteu method as described by Tongpoothorn et al. [14]. All analytical methods were performed in triplicate and the results expressed as means±standard deviations (SD).

7. Cellulosic Ethanol Fermentation

7-1. Microorganism and Inoculums Preparation

S. cerevisiae TISTR 5048 from the Microbiological Resource Center, Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani, was inoculated into yeast extract-malt extract (YM) medium containing (per L): 3 g yeast extract, 3 g malt extract, 5 g peptone and 10 g glucose. It was then incubated on a rotating shaker at 100 rpm, 30 °C for 24 h. To prepare inoculums for cellulosic ethanol production, yeast cell suspensions in the broth were quantified and adjusted to 1×10^8 cell/mL by using a haemacytometer (Sigma-Aldrich, Germany).

7-2. Fermentation Medium and Conditions

The hydrolyzate of the two-stage pretreated sample under optimum conditions (adjusted to pH 5.50) was supplemented with the following synthetic nutrients (per L): 1 g yeast extract, 1 g $MgSO_4 \cdot 7H_2O$, 2 g $(NH_4)_2SO_4$ and 5 g KH_2PO_4 [15] and used as a medium for cellulosic ethanol fermentation. This medium was transferred into a 5 L fermenter and sterilized by autoclaving at 121 °C, 15 psi for 30 min. Then, an inoculum suspension of *S. cerevisiae* TISTR 5048 cells was loaded into the sterilized medium (10% v/v). The fermentation was operated at 30 °C under static conditions for 72 h. The fermented broth was collected at 6-h time intervals for determination of cellular growth, residual glucose and ethanol concentration.

7-3. Determination of Cell Growth, Glucose and Ethanol Concentrations

After centrifugation of the fermented broth, the supernatant was analyzed for residual sugar concentrations using an HPLC equipped with an Aminex HPX-87H column according to the conditions in item 6. Cellular growth was monitored by counting the cell number in a haemacytometer under optical microscope. Cellulosic ethanol concentration (mean values and standard deviation) was determined using a gas chromatograph (GC) (TraceGC, Thermo Finnigan, Italy) with a capillary DB-5 column (30×0.25 mm i.d.) (J & W Scientific, USA) and a flame ionization detector under the following conditions: carrier gas, helium; oven temperature, 60 °C; injection temperature, 250 °C and detector temperature, 280 °C. *n*-butanol was used as an internal standard. Sample injection volume was 1 μL.

RESULTS AND DISCUSSION

1. Effect of Pretreatments on the Main Compositions of Cassava Stem

The chemical compositional change of cassava stem before and after each pretreatment is presented in Table 2. Clearly, the untreated and pretreated cassava stem differed in cellulose, hemicellulose and lignin content. Untreated cassava stem consisted of 42.10% cellulose, 14.85% hemicellulose and 35.63% lignin, and others. After acid-chlorite pretreatment, the content of lignin decreased to 4.42%, indicating that 87.59% of lignin from raw material could be removed after this pretreatment. The main effect of acid-chlorite pretreatment utilizing an aqueous solution of $NaClO_2$ and CH_3COOH is to degrade lignin from lignocellulosic biomass at moderate temperature conditions (70-80 °C) to improve the hydrolysis efficiency since lignin inhibits the accessibility of acids or enzymes to cellulose [9, 16]. After that, NaOH pretreatment was applied which reduced hemicellulose content to 3.24%, indicating that hemicellulose content decreased by 78.18% as compared to the raw material because NaOH pretreatment was effective in removing hemicellulose. Removal of hemicellulose from biomass by alkali involves hydrolysis of ester and ether linkages between hemicellulose and lignin [17].

Table 2. Chemical composition of cassava stem before and after each pretreatment

Pretreatment method	Composition (%)			
	Cellulose	Hemicellulose	Lignin	Ash
Untreated	42.10±0.15	14.85±0.07	35.63±0.19	5.97±0.03
Acid-chlorite	54.83±0.32	12.25±0.04	4.42±0.05	3.61±0.01
Acid chlorite followed by NaOH	86.45±0.53	3.24±0.02	3.05±0.01	1.56±0.01

Furthermore, two-stage pretreatment increased cellulose content to 86.45%, suggesting that cellulose is readily converted into sugars in the subsequent acid hydrolysis stage. Other two-stage pretreatments were studied to remove hemicellulose and break down the lignin barrier to enhance cellulose digestibility. Qi et al. [18] performed a two-stage pretreatment of wheat straw using NaOH followed by H_2O_2 and obtained a 65.97% delignification and 20.10% hemicellulose solubilization. Zhang et al. [6] conducted pretreatment of corn cob with dilute H_2SO_4 followed by dilute NaOH and found a loss of 81% for lignin and 66.8% for hemicellulose. Boonmanumsin et al. [7] studied microwave-assisted diluted NH_4OH followed by H_3PO_4 pretreatment of *Miscanthus sinensis* and observed 41.21% hemicellulose solubilization and 40.23% delignification, respectively. The cassava stem pretreated with the acid-chlorite followed by NaOH in this work exhibited the highest effectiveness for the removal of lignin and hemicellulose as compared to those from other works, resulting in high cellulose content in cassava stem. Generally, an increase in cellulose content in pretreated lignocellulosic biomass increased the level of fermentable sugars achieved from acid or enzymatic hydrolysis [19,20].

2. RSM Optimization of the Hydrolysis Conditions

2-1. Statistical Model Analysis

CCD for the experimental design and predicted results are shown in Table 3. The application of RSM yielded the following quadratic regression model, which was an empirical relationship between total reducing sugar yield and the studied independent variables in coded unit:

$$Y_i = 65.61 + 0.78A + 9.38B - 4.49C - 14.22A^2 - 12.37B^2 - 14.91C^2 - 4.10AB + 2.03AC - 3.47BC \quad (5)$$

where Y_i is the predicted total reducing sugar yield (g/100 g substrate), while A, B and C are the coded units of H_2SO_4 concentration, ratio of acid volume to sample and hydrolysis time, respectively.

The statistical significance of the model was evaluated using Fisher's test for analysis of variance (ANOVA) (Table 4). A model F value of 220.50 and probability value (Prob>F) less than 0.0001 imply significant model fit. The goodness of the fit of the quadratic regression model is assessed by determining the R^2 and adjusted R^2 coefficients. The R^2 value was calculated as 0.9960, meaning that the regression model can explain 99.60% of the variability in the response and only 0.40% of the total variance cannot be attributed to the independent variables. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1, the stronger the model and the better it predicts the response. Normally, a regression model with an R^2 value higher than 0.9000 is considered to have a very high correlation [18]. Fig. 1 shows the predicted total reducing sugar yield from the model versus that from the observed results. As can be seen, the observed total reducing sugar yield agrees well with the predicted results from the quadratic model in the range of the operating variables. Adjusted R^2 is a modification of R^2 that adjusts for the number of terms in a model. The value of the adjusted R^2 was 0.9915, which is also very high. These results show that the regression model provides a good fit to the data at 99.15% confidence level. The coefficient of variation (CV) affects the degree of precision with which the experiments are compared and is a good index of the reliability of the experiment. The lower the CV value, the higher is the reliability of the experiment [21]. A relatively low CV value (5.48%) clearly denotes a better precision and a good deal of reliability of the experiments. The signal (response) to noise (deviation) ratio was measured by "Adeq Precision." A ratio greater than 4 is desir-

Table 3. Experimental design with three independent variables and results obtained by hydrolysis of two-stage pretreated cassava stem

Runs	Variables						Total reducing sugar yield (g/100 g substrate)	
	Coded values			Actual values			Observed	Predicted
	A	B	C	A	B	C		
1	−1	−1	−1	0.10	10	60	14.72	12.89
2	+1	−1	−1	0.30	10	60	18.07	18.60
3	−1	0	−1	0.10	15	60	46.11	46.79
4	−1	−1	+1	0.10	10	120	5.87	6.78
5	+1	0	−1	0.30	15	60	35.91	36.09
6	+1	−1	+1	0.30	10	120	20.22	20.62
7	−1	0	+1	0.10	15	120	26.50	26.80
8	+1	0	+1	0.30	15	120	21.32	24.23
9	0	0	0	0.20	15	90	65.56	65.61
10	0	0	0	0.20	15	90	65.58	65.61
11	0	0	0	0.20	15	90	65.59	65.61
12	0	0	0	0.20	15	90	65.44	65.61
13	+1.68	0	0	0.37	15	90	28.56	26.69
14	−1.68	0	0	0.03	15	90	23.72	24.06
15	0	+1.68	0	0.20	23.41	90	48.43	46.38
16	0	−1.68	0	0.20	6.59	90	14.32	14.84
17	0	0	+1.68	0.20	15	141	18.19	15.88
18	0	0	−1.68	0.20	15	40	30.21	30.99

Table 4. ANOVA for quadratic model for total reducing sugar yield

Source	Sum of squares	Degree of freedom	Mean square	F-value	Prob>F
Model	6924.51	9	769.39	220.50	<0.0001
A	96.26	1	96.26	27.59	<0.0008
B	1201.11	1	1201.11	344.23	<0.0001
C	275.54	1	275.74	79.02	<0.0001
A^2	2559.39	1	2559.39	733.50	<0.0001
B^2	1936.68	1	1936.68	555.03	<0.0001
C^2	2812.16	1	2812.16	805.94	<0.0001
AB	134.73	1	134.73	38.61	0.0003
AC	33.09	1	33.09	9.48	0.0151
BC	8.40	1	8.40	2.41	0.1594*
Residual	27.91	8	3.49		
Lack of fit	6.12	5	1.22	0.66	0.6681*
Pure error	0.014	3	0	4.83×10^{-3}	
Core total	6952.43	17			
CV=5.48%; R^2=0.9960; Pred R^2=0.9686; Adj R^2=0.9915; Adeq precision=42.256					

*Prob F value more than 0.05 indicates that the model terms are not significant

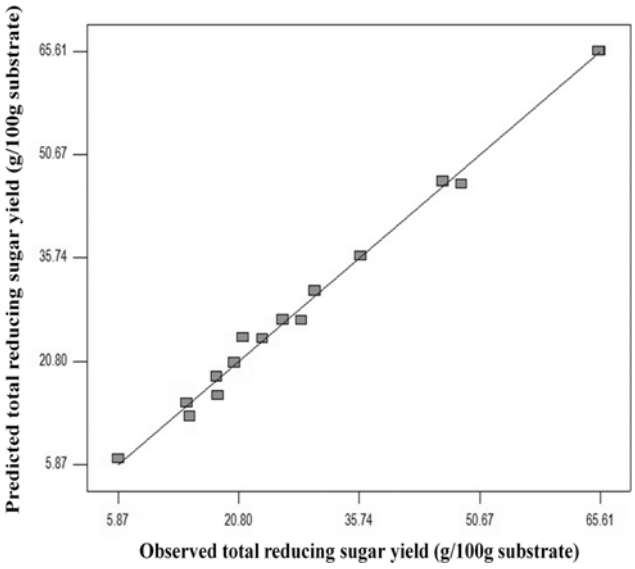

Fig. 1. Predicted total reducing sugar yield vs. the observed total reducing sugar yield under different hydrolysis conditions.

able [22]. The ratio of 42.256 obtained indicates a better precision and a good deal of reliability for the experimental values. Furthermore, the results of the error analysis indicate that the lack of fir was not significant (P=0.6681), indicating that the model is adequate to predict total reducing sugar yield within the range of experimental variables. The P-value was applied to check the significant of each coefficient (Table 4). In general, P-values less than 0.05 indicates that the model term are statistically significant, whereas a value greater than 0.10 indicates that model terms are not significant [23]. In this case, linear terms (A, B, C), quadratic terms (A^2, B^2, C^2) and cross-product terms (AB, AC, all except BC) are significant model terms (P<0.05). This suggests that H_2SO_4 concentration, ratio of acid volume to sample and hydrolysis time have a direct effect on reducing sugar yield.

2-2. Process Optimization for H_2SO_4 Hydrolysis of Pretreated Cassava Stem

The fitted regression Eq. (5) was described in form of 3D surface plots and corresponding 2D contour plots to illustrate the effects of variables and their interactions on total reducing sugar yield (Fig. 2).

Fig. 2(a) shows the response surface and contour plots for the combined effect of H_2SO_4 concentration and hydrolysis time on total reducing sugar yield. The results demonstrate that reducing sugar yield increases from 38.96 to 60.27% when H_2SO_4 concentration and hydrolysis time are increased. Then the value gradually decreases from 60.27 to 38.96% with an increase in H_2SO_4 concentration (from 0.20 to 0.30 M) and hydrolysis time (from 90 to 120 min). The trends of decreasing total reducing sugar yield at too high acid concentration and long hydrolysis time are due to the decomposition of sugars to form fermentation inhibitory compounds such as hydroxymethylfurfural, furfural, levulinic acid and acetic acid [24,25]. The maximum reducing sugar yield was achieved with H_2SO_4 concentration of 0.20 M and hydrolysis time of 90 min.

Fig. 2(b) and 2(c) show the effect of the interaction between the ratio of acid volume to sample and another variable on reducing sugar yield. The 3D response and contour lines reveal that reducing sugar yield continuously increases as the ratio of acid volume to sample increases. The highest reducing sugar yield was achieved using the ratio of acid volume to sample in the range of 15-20 mL/g while incorporating other experimental conditions.

To determine the optimum values of the studied variables for total reducing sugar yield, the regression equation (Eq. (5)) was resolved and then an overlay contour plot created using Design Expert software version 6.0.10. The optimum hydrolysis conditions to achieve high total reducing sugar yield were defined with the following criterion: total reducing sugar yield >60 g/100 g of substrate. The non-shaded area in the overlay plot in Fig. 3 is the region that meets the proposed criterion. Based on the overlay plot, the

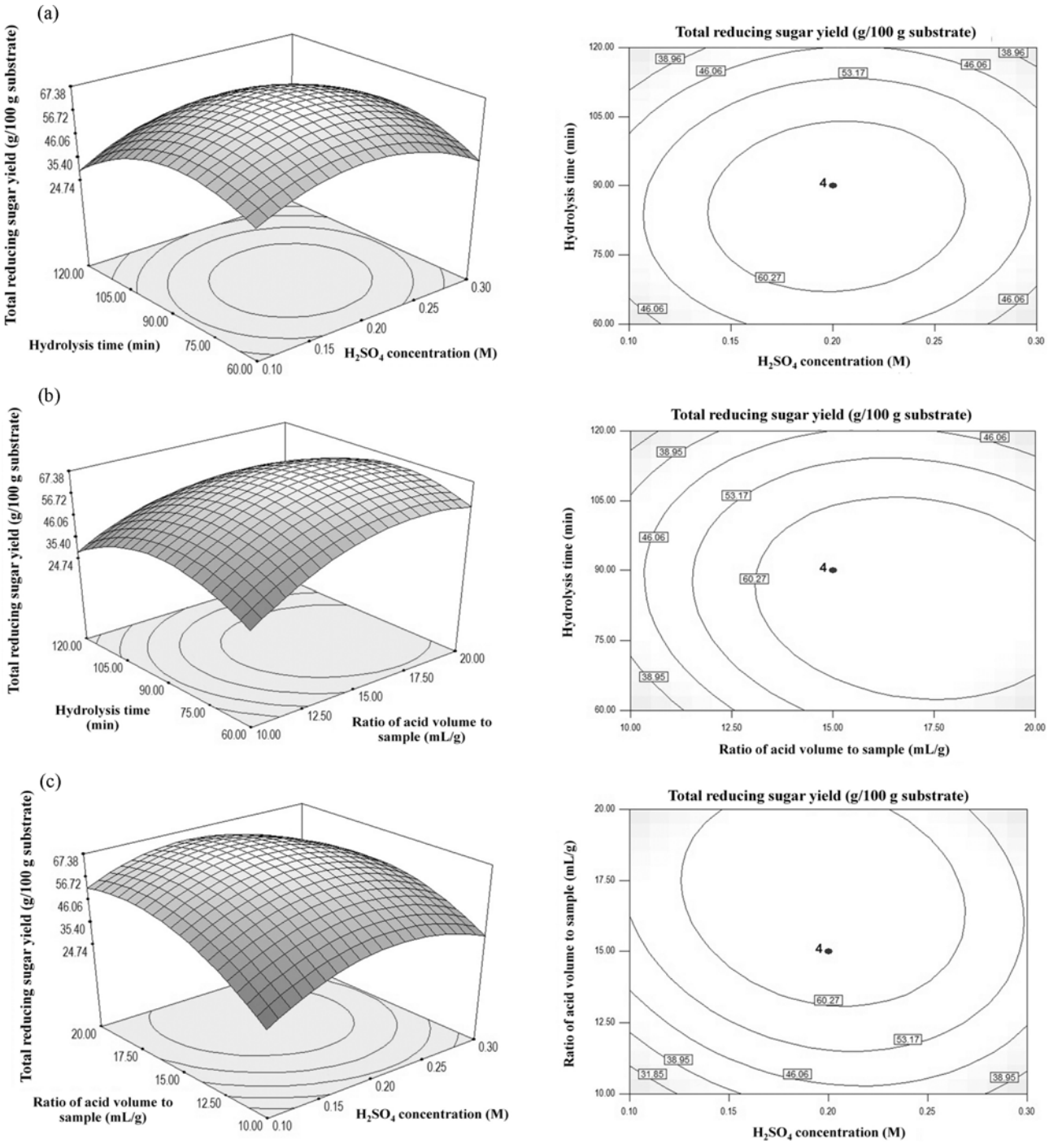

Fig. 2. (a) 3D surface plot and the corresponding contour plot showing the effect of acid concentration and hydrolysis time on total reducing sugar yield. (b) 3D surface plot and the corresponding contour plot showing the effect of ratio of acid volume to sample and hydrolysis time on total reducing sugar yield. (c) 3D surface plot and the corresponding contour plot showing the effect of acid concentration and ratio of acid volume to sample on total reducing sugar yield.

optimum hydrolysis parameters were found to be at H_2SO_4 concentration of 0.20 M, ratio of acid volume to sample of 16.71 mL/g and hydrolysis time for 90 min. The predicted maximum yield of total reducing sugar was recorded as 67.37 g/100 g substrate.

2-3. Validation of Predictive Model

A repetition of an experiment under optimum hydrolysis conditions was carried out to validate the results predicted by the regression model. The maximum experimental value of total reducing sugar yield obtained from ten replications was 67.18±0.35 g/100 g substrate. Excellent agreement between the actual and predicted values (0.28% deviation error) confirms the response model's adequacy and validity.

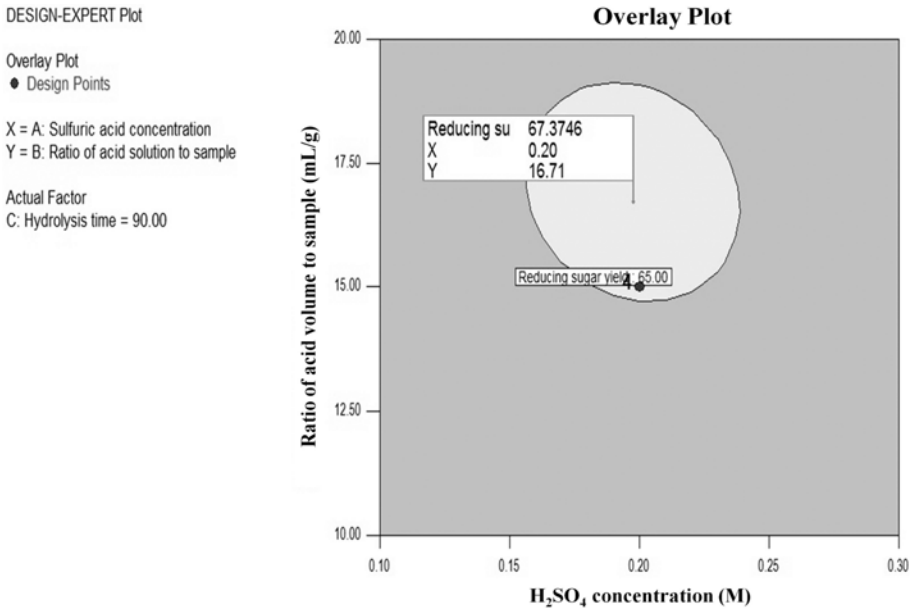

Fig. 3. Optimum region obtained by overlaying plots of the response evaluated (total reducing sugar yield) as a function of H_2SO_4 concentration, ratio of acid volume to sample and hydrolysis time.

Table 5. Composition of the hydrolysate of two-stage pretreated cassava stem obtained under optimum conditions

Compositions	Concentrations (g/100 g substrate)
Glucose	55.30±2.30 (33.11 g/L)
Xylose	9.03±0.45 (5.41 g/L)
Arabinose	1.23±0.05 (0.73 g/L)
Furfural	0.07±0.04 (0.04 g/L)
5-Hydroxymethylfurfural	0.14±0.03 (0.08 g/L)
Acetic acid	1.05±0.02 (0.63 g/L)
Phenolic compounds	0.06±0.00 (0.04 g/L)

3. Characterization and Quantification of Different Sugar Monomers and Inhibitor Compounds Present in the Hydrolyzate

Table 5 shows the composition of hydrolyzate obtained under optimum conditions. The hydrolysis conditions provided a hydrolyzate with a high concentration of glucose (55.30 g/100 g substrate) and low concentration of xylose and arabinose. This is because glucose was the main composition released from the acid hydrolysis of cellulose, which has high content in the two-stage pretreated cassava stem.

The choice of acid hydrolysis of lignocellulosic biomass may result in a variety of inhibitors. Furan derivatives (furfural and hydroxymethylfurfural), acetic acid and phenolic compounds are the prominent and most investigated inhibitors [26]. The quantities of inhibitors in the hydrolyzate of two-stage pretreated cassava stem are shown in Table 5. It was observed that inhibitors were also generated in the hydrolysis stage of lignocellulosic biomass. Furfural, 5-hydroxymethylfurfural, acetic acid and phenolic compounds were found at concentration levels of 0.06 g/L, 0.08 g/L, 0.63 g/L and 0.04 g/L, respectively. Some previous studies examined the inhibition effect of furfural and hydroxymethylfurfural on the fermentation of the acid hydrolyzate by different microorganisms. Taherzadeh et al. [27] and Karimi et al. [28] reported that 1 g/L of furfural or hydroxymethylfurfural is sufficient to inhibit cell growth and ethanol production by *S. cerevisiae*. In this experiment, furan compound concentration in the hydrolyzate was lower than the threshold considered inhibitory for yeast metabolism, indicating that these compounds do not adversely affect the efficiency of ethanol fermentation of the yeast used in this process. Acetic acid in the hydrolyzates was derived from the hydrolysis of the acetyl groups bound to the hemicellulose monomer. This acid could be an inhibitor to microbial growth because it enters the cell membrane and decreases intercellular pH, thus affecting the metabolism of the microorganisms [29]. Phenolic compounds are derived from the breakdown of lignin during acid hydrolysis. They could decrease the growth rate of microorganisms by affecting the cell membrane integrity and activities [30]. In this study, acetic acid and phenolic compounds were also detected in the hydrolyzate at very low levels. The hydrolyzate components of untreated (control) and two-stage pretreated cassava stem under optimum conditions (calculated based on g/100 g untreated cassava stem) were compared to evaluate the effectiveness of two-stage pretreatment, as shown in Fig. 4. Compared to untreated cassava stem hydrolyzate, two-stage pretreated cassava stem hydrolyzate obviously showed an increase in sugars (glucose, xylose and arabinose). In addition, fermentation inhibitor compounds (furfural, hydroxymethylfurfural, acetic acid and phenolic compounds) rarely occurred compared to untreated biomass hydrolyzate. Therefore, the acid hydrolyzate of two-stage pretreated cassava stem under optimum conditions is a promising source for producing cellulosic ethanol by *S. cerevisiae* TISTR 5048. The results verify that two-stage pretreatment is effective in improving cassava stem hydrolyzability to maximize the production rate of fermentable sugars and minimize the production of inhibitor compounds.

Table 6 presents a comparison of the acid hydrolyzate compo-

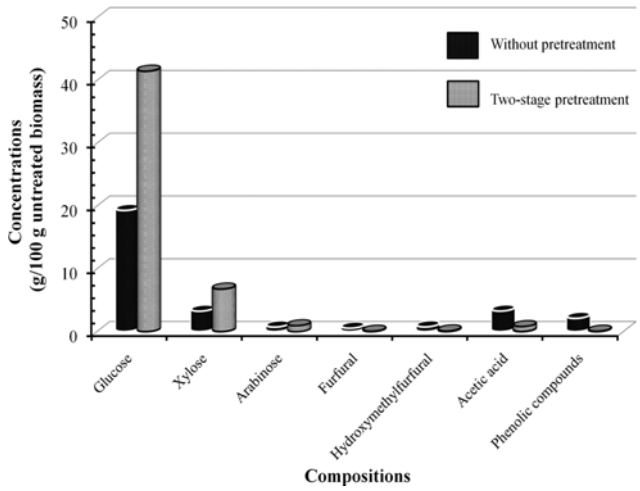

Fig. 4. Comparison of the main components of acid hydrolysates of untreated and two-stage pretreated cassava stem under optimum conditions.

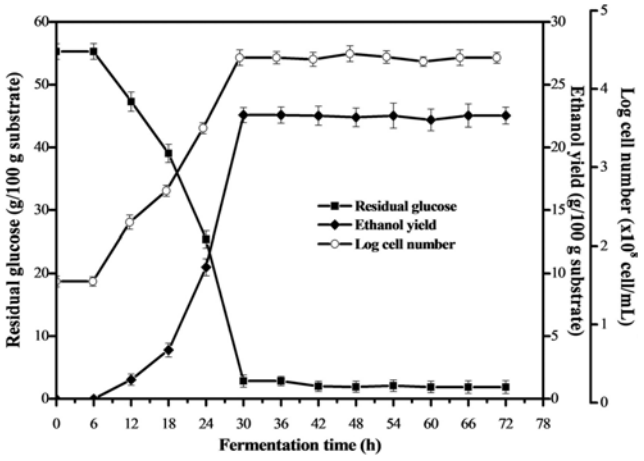

Fig. 5. Time-course profile of cellulosic ethanol fermentation by *S. cerevisiae* TISTR 5048 from the hydrolysate under optimized hydrolysis conditions.

nents of various pretreated lignocellulosic biomasses. The quantities of total monomeric sugars and glucose obtained in this work were the highest when compared with those from other works. Total inhibitor compounds detected in this work also occurred less than in other works. Therefore, removal of inhibitor compounds from the acid hydrolyzate was not essential. In some studies, inhibitor compounds such as furan derivatives, acetic acid and phenolic compounds present in the hydrolyzate were removed using polyelectrolyte polyethyleneimine [31], electrodialysis [32] or a surfactant-based aqueous two phase system [33] before the hydrolysate could be used as a source for bioconversion to ethanol. This causes an increase in the production cost of lignocellulosic biomass hydrolysis.

4. Cellulosic Ethanol Fermentation from the Hydrolysate by *S. cerevisiae* TISTR 5048

Fig. 5 demonstrates the time course profiles and characteristic of ethanol fermentation using *S. cerevisiae* TISTR 5048 from hydrolyzate under the appropriate conditions (initial glucose concentra-

Table 6. Comparison of the main components of the acid hydrolysates of various pretreated lignocellulosic biomasses from several works

Lignocellulosic biomass	Optimum acid hydrolysis conditions	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Furan compounds (g/L)[a]	Acetic acid (g/L)	Phenolic compounds (g/L)	References
Wheat straw	1.85% H_2SO_4, solid : liquid of 1 : 20, 90 °C, 18 h	1.70	12.80	2.60	0.15	2.70	-	Nigam [34]
Rice straw	1.60% H_2SO_4, solid : liquid of 1 : 10, 121 °C, 30 min	20.50	6.30	3.00	0.31	2.00	-	Roberto et al. [35]
Sugarcane bagasse	4% H_3PO_4, 122 °C, 5 h	3.00	17.60	2.60	1.20	4.00	-	Gámez et al. [36]
Rice hulls	0.50% H_2SO_4, solid : liquid of 1 : 10, ambient temperature, 24 h and 190 °C, 10 min	10.00	11.00	-	0.13	-	-	López et al. [37]
Corn stover	1.69% H_2SO_4, solid : liquid of 1 : 10, 121 °C, 117 min	1.85	9.11	0.88	0.41	3.19	0.04	Cao et al. [22]
Olive tree	1% H_2SO_4, solid : liquid of 1 : 100, 190 °C, 10 min	21.70	16.40	-	3.42	6.81	-	Díaz et al. [38]
Corncob	0.50% H_2SO_4, solid : liquid of 1 : 10, 125 °C, 90 min	2.51	28.55	3.17	0.23	3.74	-	Cai et al. [39]
Cassava stem	0.20 M (1.07%) H_2SO_4, solid : liquid of 1 : 16.70, 121 °C, 90 min	33.11	5.41	0.44	0.12	0.63	0.03	This work

A dash (–) indicated no reported

[a]Each value is expressed as the sum of furfural and hydroxymethylfurfural concentrations

tion of 55.30 g/100 g substrate), including the number of yeast cells, residual glucose and ethanol yield. After a short lag phase of 6 h, cell growth started immediately and the logarithmic phase of growth was visualized at 12-30 h. Cellulosic ethanol production began in the early exponential phase of yeast growth and remained at high production level until the late exponential growth phase. The time course profile of ethanol fermentation obviously shows that the glucose concentrations declined gradually as fermentation proceeded, while xylose and arabinose had no significant change (data not shown). The lowest glucose concentration of 2.85 g/100 g substrate was reached at 30 h of fermentation time, and the highest ethanol yield based on glucose utilization was 22.58 g/100 g substrate. The results indicate that 52.45 g glucose was utilized at the end of fermentation.

5. Ethanol Yield and Productivity

The ethanol productivity was calculated according to the following Eq. (6):

$$Q_p = \frac{P}{t} \tag{6}$$

where Q_p is the ethanol productivity (g/100 g/h), P is the actual ethanol yield produced (g/100 g) and t is the fermentation time (h) giving the maximum ethanol yield.

The percentage of conversion efficiency of glucose to ethanol or theoretical ethanol yield is calculated, assuming that 1 g of glucose present in the hydrolyzate would theoretically give 0.51 g of ethanol. Theoretical ethanol yield was calculated according to Eq. (7):

$$T_y = \frac{P \times 100}{G \times 0.51} \tag{7}$$

where T_y is the theoretical ethanol yield (%), P is the ethanol yield (g/100 g substrate), G is the glucose utilized (g/100 g substrate) and 0.51 is the theoretical conversion factor for ethanol from glucose by *S. cerevisiae*.

Ethanol yields and productivities obtained by calculating of the results in this work and the literature review are compared in Table 7. The ethanol yield and ethanol productivity obtained from cassava stem in this work was highest as compared to those from other lignocellulosic substrates. Compared to the same lignocellulosic substrate type, the theoretical yield of ethanol based on glucose utili-

Table 7. Comparison of cellulosic ethanol yields and productivities calculated from the results in this work and the literature reviews

Lignocellulosic biomass	Hydrolysis process	Fermentation process	EtOH conc.[a] (g/L)	EtOH yield[a] (g/100 g)	EtOH productivity[a] (g/100 g/h)	Theoretical EtOH yield (%)	References
Brewer's spent grain[b]	Enzymatic hydrolysis by cellulase and hemicellulase	Solid-state fermentation by *Neurospora crassa* DSM 1129	-	7.40	0.052	36.00	Xiros et al. [40]
Olive tree[c]	H_2SO_4 hydrolysis	Co-fermentation by *S. cerevisiae* and *Pichia stipitis*	7.10	0.13	0.06	27.00	Fonseca et al. [41]
Switch grass[c]	H_2SO_4 hydrolysis	Batch fermentation by *S. cerevisiae* ATCC 2485	-	8.30	-	60	Yang et al. [42]
Napier grass[c]	NP	Direct conversion by *Klebsiella oxytoca* THLC 0409	0.46	8.20	0.036	-	Lin et al. [43]
Cassava stem[e]	Enzymatic hydrolysis by cellulase and β-glucosidase	Batch fermentation by *S. cerevisiae* CHY 1011	7.55	-	-	89.60	Han et al. [4]
Sugarcane bagasse[d]	H_2SO_4 hydrolysis	Batch fermentation by *S. cerevisiae*	8.11	17.00	-	69.06	Velmuruga and Muthukumr [44]
Water hyacinth[b]	Enzymatic hydrolysis by cellulase	Batch fermentation by *S. cerevisiae* MTCC 174	4.30	-	-	45	Singh and Bishnoi [45]
Cassava stem[f]	H_2SO_4 hydrolysis	Batch fermentation by *S. cerevisiae* TISTR 5048	13.52	22.58	0.75	84.41	In this study

A dash (−) indicated no reported
[a]Maximum values estimated
[b]Alkali (NaOH) pretreatment
[c]Untreated materials
[d]Sono-assisted alkali pretreatment
[e]Dilute H_2SO_4 pretreatment
[f]Acid-chlorite followed by NaOH pretreatment
NP=Not performed

zation was not different from other works as reported by Han et al. [4]. In this study, the hydrolysis of two-stage pretreated cassava stem was performed using dilute H_2SO_4 further fermented to ethanol using *S. cerevisiae* TISTR 5048, and it was found that the ethanol yield of 22.58 g/100 g substrate corresponded to a theoretical ethanol yield of 84.41%. Han et al. [4] reported that the enzymatic hydrolysis of dilute H_2SO_4 pretreated cassava stem using a commercial cellulase combined with β-glucosidase and subsequently fermented by *S. cerevisiae* CHY 1011 produced an ethanol yield of 7.55 g/L corresponding to a theoretical ethanol yield of 89.60%. Although the theoretical ethanol yield was not different from that previously reported by Han et al. [4], the cost of acids such as H_2SO_4 used for this acid hydrolysis is much lower than commercial cellulolytic enzymes (cellulase and β-glucosidase). Therefore, this chemical hydrolysis is more economically viable for large-scale ethanol production from lignocellulosic biomass than the bioconversion process. Moreover, the optimum conditions of H_2SO_4 hydrolysis derived via RSM were used under low concentration conditions (0.20 M), which may give rise to two advantages of the processes, low toxicity of the hydrolyzate and low utility cost, since there are low corrosion problems, leading to cost reduction for large-scale industrial production of ethanol.

CONCLUSIONS

After two-stage chemical pretreatment, the cellulose content of cassava stem increased from 42.10% of raw material to 86.45%, concomitant with decreases in lignin and hemicellulose contents. Acid hydrolysis of two-stage pretreated cassava stem under optimum conditions derived via RSM allowed the production of hydrolyzate rich in reducing sugar, whereas fermentation inhibitor compounds (furan derivatives, acetic acid and phenolic compounds) were detected at very low concentrations. The yield of fermentable sugars in two-stage pretreated cassava stem hydrolyzate was significantly higher as compared untreated material hydrolyzate. These findings indicate that two-stage pretreatment is effective for the removal of lignin and hemicellulose matrix, resulting in high cellulose content in the substrate, which facilitated the subsequent dilute H_2SO_4 hydrolysis to obtain high fermentable sugar and low by-product concentrations. Cellulosic ethanol yield and productivity were measured to be 22.58 g/100 g substrate, 0.75 g/100 g/h, respectively, and the highest compared to those from other works. Therefore, the acid hydrolyzate of cellulose fraction of two-stage pretreated cassava stem under the appropriate conditions containing highly fermentable sugar and low inhibitor concentrations is a promising source for high yield cellulosic ethanol production.

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