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Bioethanol production from optimized pretreatment of cassava stem

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Abstract−The current ethanol production processes using crops such as corn and sugar cane are well established. However, the utilization of cheaper biomasses such as lignocellulose could make bioethanol more competitive with fossil fuels, without the ethical concerns associated with the use of potential food resources. A cassava stem, a lignocellulosic biomass, was pretreated using dilute acid to produce bioethanol. The pretreatment conditions were evaluated using response surface methodology (RSM). As a result, the optimal conditions were 177 °C, 10 min and 0.14 M for the temperature, reaction time and acid concentration, respectively. The enzymatic digestibility of the pretreated cassava stem was examined at various enzyme loadings (10-40 FPU/g cellulose of cellulase and 30 CbU/g of β-glucosidase). With respect to economic feasibility, 20 FPU/g cellulose of cellulase and 30 CbU/g of β-glucosidase were selected for the test concentration and led to a saccharification yield of 70%. The fermentation of the hydrolyzed cassava stem using *Saccharomyces cerevisiae* resulted in an ethanol concentration of 7.55 g/L and a theoretical fermentation yield of 89.6%. This study made a significant contribution to the production of bioethanol from a cassava stem. Although the maximum ethanol concentration was low, an economically efficient overall process was carried out to convert a lignocellulosic biomass to bioethanol.

Key words: Bioethanol, Response Surface Methodology (RSM), Cassava Stem, Enzymatic Hydrolysis, Fermentation

INTRODUCTION

During the last few decades, the excessive consumption of fossil fuels has led to an increasing demand for alternative sources of fuel [1]. These alternative sources may rely upon the production of renewable energy sources, such as ethanol. Currently, ethanol is mainly produced from sugar or starch for the fuel industry. However, the demand for these raw materials, which are also food sources, will not be sufficient to meet the need for fuel ethanol [2]. Cellulosic ethanol is one of the most promising technological approaches available to reduce the emission of greenhouse gases from the transportation sector [3]. Also, lignocellulosic biomass is of a low cost feedstock that is widely available and does not involve the ethical concerns associated with the use of potential food resources [4]. For this reason, the development of a process to convert lignocellulosic biomass into ethanol is imperative. The conversion of lignocellulosic biomass to ethanol is, however, more challenging because of the complex structure of the plant cell wall. Pretreatment is required to alter the structural and chemical composition of the lignocellulosic biomass to facilitate rapid and efficient hydrolysis of the carbohydrates to fermentable sugars [5].

A variety of physical (comminution, hydrothermolysis), chemical (acid, alkali, solvents, ozone), physico-chemical (steam explosion, ammonia fiber explosion), and biological pretreatment techniques have been developed to improve the accessibility of the enzymes to the cellulosic fibers [6]. Acid pretreatment indicates the use of sulfuric, nitric, or hydrochloric acids to remove the hemicellulose components and expose cellulose for enzymatic digestion [7]. Agricultural residues such as corncobs and stovers are particularly well suited for dilute acid pretreatment [8]. Alkali pretreatment refers to the application of alkaline solutions to remove lignin and various uronic acid substitutions on hemicellulose that lower the accessibility of the enzyme to the hemicellulose and cellulose [5]. Generally, alkaline pretreatment is more effective for agricultural residues and herbaceous crops than wood materials [9]. Peroxide pretreatment enhances enzymatic conversion through oxidative delignification and reduction of the cellulose crystallinity [10]. Increased lignin solubilization and cellulose availability were observed during the peroxide pretreatment of wheat straw [11], Douglas fir [12], and oak [13]. Ozonation is another attractive pretreatment method that does not leave strong acidic, basic, or toxic residues in the treated material [14]. The effect of ozone pretreatment is essentially limited to the lignin degradation. Hemicellulose is slightly attacked, while cellulose is barely affected [15]. Ozonation has been widely used to reduce the lignin content of both agricultural and forestry wastes [14].

Currently, cassava is being widely cultivated in the world, especially South-east Asia, because it contains abundant nutrients and rapidly grows in marginal land. The increase in cassava plantation is highly beneficial for economic development. Nevertheless, the disposal of left-over cassava in the field has raised concerns. The cassava stem, which mainly consists of lignocellulose, could potentially serve as a low-cost feedstock to increase the production of fuel ethanol through proper pretreatment, hydrolysis, and fermentation.

To fully utilize the cassava stem as a feedstock for ethanol production, pretreatment is required to render the cellulose fibers more amenable to the action of the hydrolytic enzymes. Response surface methodology (RSM) was used to determine the optimal pre-

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treatment conditions in order to save money and energy. RSM is a statistical technique used to model and optimize multiple variables that determines the optimum process conditions by combining experimental designs with interpolation of first- or second-order polynomial equations in a sequential testing procedure. This methodology has already been successfully applied for the optimization of the enzymatic hydrolysis of several substrates including cellulose [16-21]. According to these conditions, saccharification was performed using the cellulase complex, and finally, bioethanol was produced using the fermentation process.

MATERIALS AND METHODS

1. Biomass and Enzymes

Cassava stems, cultivated in the summer of 2008, were obtained from the Changhae cassava farm in Papua New Guinea. Prior to the composition analysis, the biomass was ground to a particle size of less than 1 mm in a cutter mill and stored in sealed plastic bags at room temperature until it was used. Fig. 1 shows the structures of the major components of the cassava stem (a: cellulose; b: hemicellulose; c: lignin). Lignin, a three-dimensional amorphous polymer based on methoxylated phenylpropanoid units, is covalently bonded to the carbohydrates in the plant and therefore crosslinks to different polysaccharides, which confers mechanical strength to the cell wall [22]. Dissolution and further separation were difficult because of the complex matrix of carbohydrates inside the plant cell walls.

The enzymes were provided by Novozymes in Denmark. A cellulase complex (NS50013) and β-glucosidase (NS50015) were used to investigate the enzymatic digestibility. Table 1 summarizes the characteristics of these enzymes. All of the reagents used in this study were of analytical grade.

Table 1. Characteristics of the enzymes used in this study

Enzyme (Name)	Activities*	Density (g/mL)	pH (-)	Temp. (°C)
NS50013 (Cellulase complex)	700 EGU/g (approx. 70 FPU/g)	1.2	4.5-6.5	45-50
NS50010 (β-glucosidase)	250 CbU/g	1.2	2.5-6.5	45-70

*EGU=Endo-Glucanase, FPU=Filter Paper Unit and CbU=Cellobiase Unit

2. Dilute Acid Pretreatment and Enzymatic Hydrolysis

In this study, 4.5 mL of dilute acid solution was used to pretreat 0.5 g of ground cassava stem samples at a solid loading of 10% (w/v). The treatments were performed at various temperatures and times in an oil bath. The reaction time was estimated after approaching a set temperature. After cooling, the solid and liquid fractions of the treated biomass were collected by washing the samples with distilled water to minimize the loss of the biomass. Finally, the biomass concentration was adjusted to 5% (w/v). The pH was subsequently adjusted to 5-6 and the biomass was then used for enzymatic hydrolysis.

Two enzyme solutions, cellulase complex and β-glucosidase, were used to investigate the influence of the enzyme concentration on the enzymatic hydrolysis. The hydrolysis was conducted at a cellulase activity of 10-40 FPU/g cellulose and a β-glucosidase activity of 30 CbU/g at 50 °C and 120 rpm for 60 h. After the reaction, 1.0 mL of aliquots was taken and centrifuged at 5,000 rpm for 10 min. The supernatant was removed for the sugar content analysis [23]. The percentage of glucan conversion was calculated with the following equation:

$$\%\ \text{glucan conversion} = \frac{\%GH}{\%GP} \times 100 \quad (1)$$

In this equation, GH is the dry-weight percentage of glucose in the enzyme hydrolysis supernatant (g glucose/g solids hydrolyzed %),

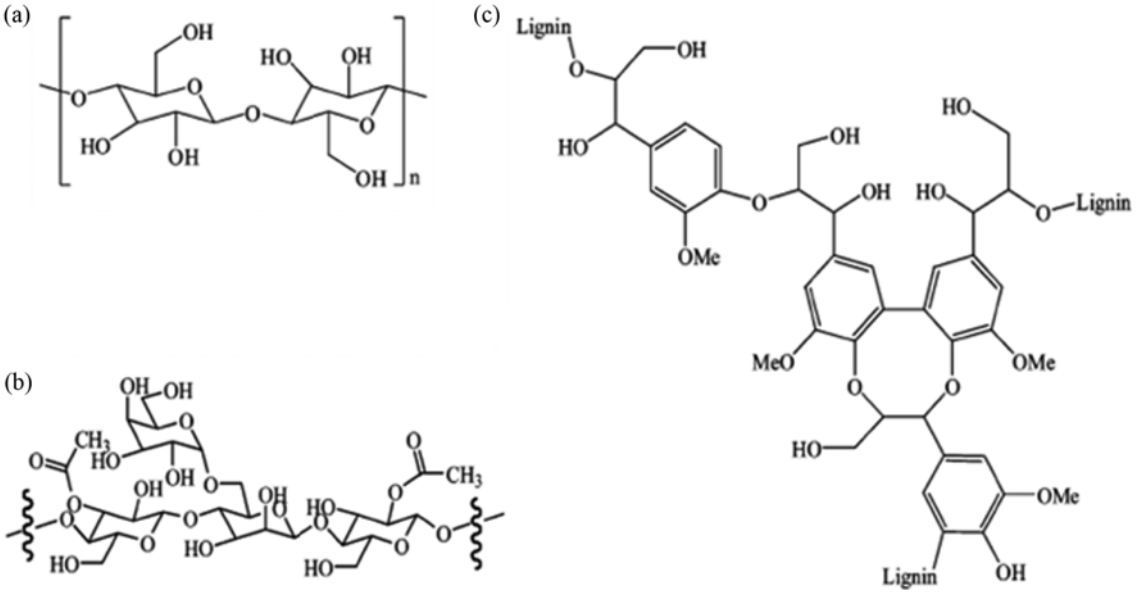

Fig. 1. Major components of the cassava stem; (a) cellulose, (b) representative structure of hemicellulose (galactoglucomannan, major hemicellulose in wood), (c) representative structure of lignin.

Table 2. Coded and decoded values for each variable of the central composite rotatable design

Coded levels of the experimental factors	X_1: Temperature (°C)	X_2: Time (min)	X_3: Acid concentration (M)
−2	146.4	1.6	0.07
−1	160.0	5.0	0.10
0	180.0	10.0	0.15
1	200.0	15.0	0.20
2	213.6	18.4	0.23

and GP is the dry-weight percentage of glucan in the pretreated solids (g glucose/g solids pretreated %).

3. Experimental Design

RSM is a collection of mathematical and statistical techniques that are useful for modeling and analyzing problems where the response of interest is influenced by several variables, and the objective is to optimize this response [24]. Many variables could potentially affect the efficiency of the pretreatment process.

In this study, a central composite rotatable design (CCRD) was employed to determine the effects of independent variables on the response and factor interactions, with different combinations of variables. Three independent variables, namely the temperature (X_1), reaction time (X_2) and acid concentration (X_3), were studied at three levels with three repetitions at the central point an two replicates at the axial and factorial points (Table 2).

For each of the five variables studied, high (coded +1) and low (coded −1) set points were selected according to the results obtained in preliminary tests. The results of each CCRD were analyzed by using the Design Expert® software version 7.1.3, from Stat-Ease, Inc., Minneapolis, USA. Both the linear and quadratic effects of the five variables were calculated, as well as their possible interactions with the released mass of glucose from the dry biomass. The significance of these variables was evaluated by variance analysis (ANOVA).

Three-dimensional surface plots were drawn to illustrate the effects of the independent variables on the dependent variables, described by a quadratic polynomial equation that was fitted to the experimental data. The fit of the models was evaluated by determining the *R*-squared coefficient and the adjusted *R*-squared coefficient. For the validation of the models, optimum values for the selected variables were obtained by solving the regression equation using the Design Expert® software version 7.1.3.

4. Fermentation with Industrial Microorganism *Saccharomyces cerevisiae* CHY 1011

To properly evaluate the fermentation process, 135 mL of a dilute acid solution was used to pretreat 15 g of the ground cassava stem samples at a solid loading of 10% (w/v), and then the dilute acid pretreatment and enzymatic hydrolysis methods were carried out as described above. After saccharification, 10 g/L of yeast extract and 20 g/L of peptone were added to supply a nitrogen source and a minor nutrient, optionally. The bottles were capped and autoclaved for 15 min at 121 °C. After cooling, *S. cerevisiae* CHY 1011 was inoculated and the solid caps were replaced with caps containing silicone septa, through which 22 g needles had been pierced to exhaust CO_2 that was released during the fermentation. The bottles were then placed back on the shaker/incubator, and the temperature was reset to 32 °C. These bottles were sampled periodically for the next 30 h, and the ethanol concentration was estimated.

The *S. cerevisiae* inoculum was prepared by growing strain CHY 1011 on a solid YPD, containing 10 g yeast extract, 20 g protease peptone, and 10 g dextrose per liter supplemented with 15 g Bacto agar. The solid culture was incubated at 32 °C for 48 h, and a single colony was transferred to a 50 mL Erlenmeyer flask containing 10 mL of YPD. Then the colony was grown at 32 °C with agitation (120 rpm) for 12 h. This culture was used to inoculate the seed culture, which consisted of 200 mL of YPD in a 500 mL Erlenmeyer flask incubated for 12 h.

5. Analytical Methods

The total solids, acid soluble lignin, and acid insoluble lignin contents of the untreated cassava stem were determined by the National Renewable Energy Laboratory (NREL) using Standard Biomass Analytical Procedures [25]. The carbohydrate content of the untreated cassava stem was estimated by measuring the hemicellulose (xylan, galactan, and arabinan) and cellulose (glucan) derived sugars. The composition of the hydrolysate from the enzymatic hydrolysis was determined by measuring glucose and xylose using high performance liquid chromatography (HPLC).

The HPLC (Waters, USA) system was equipped with a Bio-Rad Aminex HPX-87P column, a guard column, an automated sampler, a gradient pump, and a refractive index detector. The mobile phase was deionized water at a flow rate of 0.6 mL/min at 85 °C. Prior to the HPLC injection, all of the samples (derived from solids and hydrolysate) were neutralized with calcium carbonate, centrifuged at 5,000 g for 10 min, and filtered through 0.2 μm syringe filters. The concentration and impurities of ethanol were determined with a Density/Specific Gravity Meter (DA-510, KEM Co, Ltd., Japan) and gas chromatography (GC) with a Supelco 6.6% CARBOWAX 20M column, Agilent, USA.

RESULTS AND DISCUSSION

1. Characteristics of Cassava Stem

The chemical composition of the cassava stem varies according to the growing location, season, harvesting methods as well as the analysis procedures [25]. The composition of the cassava stem used in this study is listed in Table 3.

Based on the HPLC carbohydrate analysis, the sugar fraction was 59.5% and the lignin fraction was 33.8% for the dry biomass. Glucan, which was derived from both the cassava stem fiber and the plant cell wall, was the major component (35.2%). Xylan, which

Table 3. Major components of the cassava stem

Component*	[%]
Cellulose	35.2
Hemicellulose	24.3
Acid-insoluble lignin	30.0
Acid-soluble lignin	3.8
Ash	2.2
Etc.	3.5

*Data in the table based on dry samples

Table 4. ANOVA results for the response from enzymatic hydrolysis

Source	Sum of squares	d.f.	Mean of square	F-value	P-value (Prob>F)
Model	501.519	9	55.724	13.908	0.0011
X_1-temp.	26.776	1	26.776	6.683	0.0362
X_2-time	1.321	1	1.321	0.330	0.5838
X_3-acid conc.	7.567	1	7.567	1.889	0.2117
X_1X_2	103.394	1	103.394	25.806	0.0014
X_1X_3	10.471	1	10.471	2.613	0.1500
X_2X_3	9.724	1	9.724	2.427	0.1632
X_1^2	324.111	1	324.111	80.894	<0.0001
X_2^2	82.345	1	82.345	20.552	0.0027
X_3^2	59.331	1	59.331	14.808	0.0063
Residual	28.046	7	4.007		
Lack of fit	27.987	5	5.597	188.236	0.0053
Pure error	0.059	2	0.030		
Total	529.565	16			

R^2=0.9470; adjusted R^2=0.8789; d.f.=degree of freedom

was the major hemicellulose constituent, constituted up to 24.3%. Lignin is a complex chemical compound most commonly derived from biomass to protect the biomass from being attacked by the enzymes. Arabinan accounted for only a small portion (>1%) of the biomass, while galatan and mannan were not detected. Additionally, the cassava stem contained a little ash and unknown components. Glucan and xylan can be converted to ethanol using pentose and hexose fermentable organisms. However, getting the microorganism to digest pentose is difficult, and therefore, cellulose was retained during pretreatment, and was used to ferment hexoses derived from the biomass in this study.

2. Optimization of Enzymatic Hydrolysis with Central Composite Rotatable Design (CCRD)

The enzymatic response was evaluated as a function of the temperature, time and acid concentration. Five sets of CCRD experiments were carried out in order to find the optimal conditions. The temperature ranged from 160 to 200 ºC, and the acid concentration ranged from 0.1 to 0.2 M in the optimal CCRD test.

A variance analysis (ANOVA) was performed to evaluate the effects of the variables and their possible interactions. The coefficients of the full model were evaluated using regression analysis, and their significance was tested. The insignificant coefficients were excluded from the model using a backward elimination. The variance analysis performed on the reduced models (Table 4) demonstrated that the models were statistically valid with *p*-values lower than 0.0011. The temperature had the lowest *p*-values (0.032) among the factors, which meant that temperature was the dominant factor affecting the enzymatic hydrolysis [27]. For interactions between the factors, the {temperature×time} *p*-value was 0.0014 and exerted the greatest influence upon the enzymatic hydrolysis. Eq. (2) describes the correlation between the significant variables and the glucose releasing rate for the pretreated biomass in terms of the decoded values.

$$\text{Glucose conc.} = -526.98132 + 5.28681\,X_1 + 9.23247\,X_2 + 510.4332\,X_3 - 0.035950\,X_1X_2 - 1.14404\,X_1X_3 - 4.41007\,X_2X_3 - 0.013405\,X_1^2 - 0.10811\,X_2^2 - 917.64373\,X_3^2 \quad (2)$$

X_1: temperature, X_2: time and X_3: acid concentration.

The relationship between the response and variables was visualized by using the response surface or contour plots. The quadratic polynomial equations (Eq. (2)) were described with the response surface plots to estimate the released glucose through enzymatic hydrolysis as a function of two factors, maintaining all of the other factors at fixed level of zero (Fig. 2).

The proportion of the total variation attributed to each fit was evaluated using the *R*-squared value (an *R*-square value >0.75 indicates a suitable model) [26]. For the pretreated cassava stem, the regression equation resulted in an *R*-squared value of 0.947, which was in good agreement with the adjusted *R*-squared of 0.879. These results ensured that the theoretical values were satisfactorily adjusted to the experimental data using this model. Therefore, the model was suitable for predicting the enzymatic hydrolysis.

The optimum values of the selected variables were obtained by solving the regression equation, as shown in Table 5. To validate the model, the optimum values for Eq. (1) were used in triplicate sets of experiments, and the maximum response obtained for each shrub is presented in Table 5. The experimental response for the cassava stem was 25.19%. This value was in good agreement with the predicted value of 25.69% (24.41-26.97%) with a 95% confidence interval. This behavior showed that the model could be adapted to the experimental results, confirming the validity and adequacy of the models.

3. Enzymatic Hydrolysis of Pretreated Cassava Stem

The enzymatic hydrolysis experiments were performed by using the pretreated biomass based on the results derived from the RSM to determine the enzyme concentration. Fig. 3 shows the enzymatic digestibility of the pretreated cassava stem with cellulase complex and β-glucosidase enzyme loadings of 10-40 FPU/g cellulose and 30 CbU/g, respectively. The conversion ratio was enhanced in accordance with an increase in enzyme dosage. Due to economic feasibility, 20 FPU/g cellulose and 30 CbU/g of cellulase (the enzy-

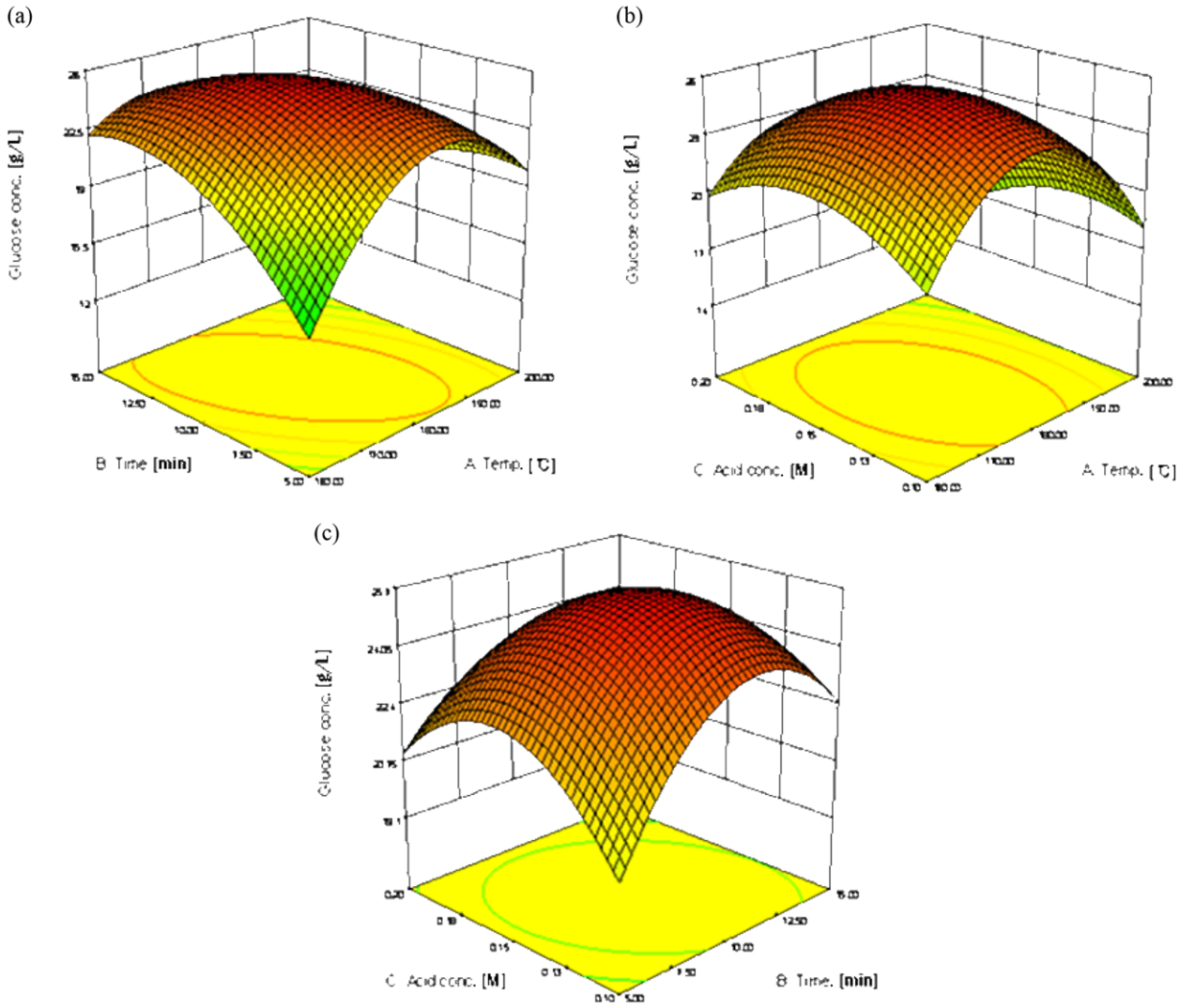

Fig. 2. Response surface plots of the central composite design for the optimization of the enzymatic hydrolysis. Effect of (a) temperature and time, (b) temperature and acid concentration, (c) time and acid concentration. Other factors were held constant at zero levels.

Table 5. Optimal values of the test variables in decoded units and the predicted maximum for a 95% confidence interval of the released mass of glucose from a dry biomass

Variables	Value
X_1: temperature (°C)	177.3
X_2: time (min)	10.0
X_3: acid concentration (M)	0.14
Predicted response with 95% confidence	25.69
interval (% of glucose/dry biomass)	(24.41-26.97)
Experimental response (% of glucose/dry biomass)	25.19±0.53

matic digestibility was approximately 70%) were used for the enzymatic hydrolysis.

4. Fermentation of Pretreated Cassava Stem with *S. cerevisiae*

The fermentabilities of the pretreated materials were evaluated using *S. cerevisiae*. The pretreated biomass was mixed with the cellulase complex and β-glucosidase for 60 h at 50 °C. Pectinase and xylanase were not included because *S. cerevisiae* does not have the

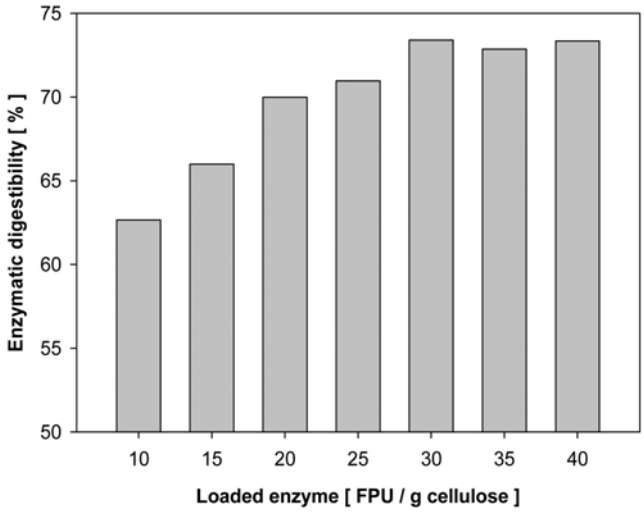

Fig. 3. Glucan conversion after enzymatic hydrolysis using the pretreated (optimal condition) cassava stem (Enzyme loading 10-40 FPU/g cellulose and β-glucosidase 30 CbU/g).

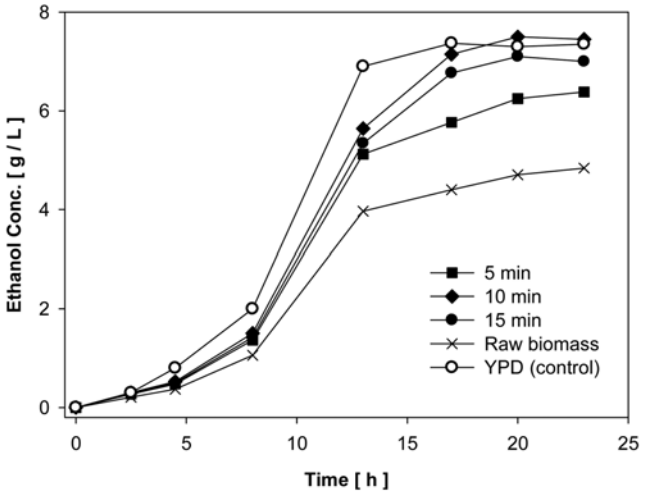

Fig. 4. Ethanol fermentation of the raw cassava stem, YPD control and pretreated cassava stem at various pretreatment reaction times (pretreatment reaction time: ■: 5 min, ◆: 10 min, ●: 15 and ×: no pretreatment, ○: YPD).

ability to digest arabinose or xylose. Fermentation was subsequently carried out for 24 h at 32 ºC.

The fermentation was performed increasing 20 times biomass loading than pretreatment for proper ethanol concentration analysis. The reaction time was estimated at 10 min for the pretreatment process from the results based on the RSM. But, the reaction time could be different as biomass loading and is shown in Fig. 4. There is no effect of reaction time with increasing biomass loading. And, as the reaction time increased, the ethanol concentration accordingly was enhanced. When the reaction time was 10 min (◆), the ethanol concentration was 7.55 g/L, which was over 1.54 times higher than that of the sample without pretreatment (4.89 g/L). However, after 15 min (▲), the ethanol concentration decreased (7.18 g/L), indicating that the ethanol product was suitable for enzymatic hydrolysis after excessive dissolution of cellulose if the pretreatment time exceeded the proper time limit.

Hydrolysate made from lignocellulosic biomass is well known to generate various inhibitors during the ethanol production process. The YPD medium (10 g/L of yeast extract and 20 g/L of peptone), which consisted of the same amount of glucose as the hydrolyzed cassava stem by enzyme, was used as a control (O). The fermentations were completed within 24 h, and the cassava stem had a slower fermentation time than YPD. In other words, inhibitors were insignificantly generated in the pretreatment process. The fermentation rate (Table 6) provided further support for this hypothesis. In general, when fermentation rate reached approximately 90%, inhibitors were not created. YPD was used as a control in this experiment and had a fermentation rate of 91%. The experiment with a reaction time of 10 min, and the highest ethanol concentration had a fermentation yield of 89.6%, which was almost the same fermentation rate as YPD. Therefore, a variety of inhibitors were not produced at a low concentration of the dilute acid in the study, even though it is generally known that fermentation inhibitors are created when a dilute acid is used for pretreatment.

CONCLUSIONS

The overall process starting from the pretreatment of the cassava stems to the production of bioethanol was examined in this study. The pretreatment process was essential to the production of ethanol from lignocellulosic biomass, which was achieved through saccharification by breaking the structure tangled with cellulose, hemicelluloses and lignin so that the enzyme could easily permeate into cellulose and hemicelluloses. In this study, dilute acid was used for pretreatment, and the optimal pretreatment conditions were obtained through the RSM. The result revealed that the optimal temperature was 177 ºC with a reaction time of 10 min and acid concentration of 0.14 M. After pretreatment, 20 FPU/g cellulose of the cellulase complex and 30 CbU/g of β-glucosidase were added and mixed at 120 rpm and 50 ºC for 60 h. Subsequently, ethanol was produced though 24 h of fermentation at 32 ºC and 120 rpm following the sterilization and inoculation of S. *cerevisiae*. The ethanol concentration was 7.55 g/L, which was relatively lower than the starch and sugar materials. Nevertheless, the results were quite interesting considering that ethanol was manufactured from cassava stem. Moreover, the fermentation yield was 89.6%, which was no less than the yield of YPD (control). Therefore, the production of bioethanol for commercial use could be feasible in the near future.

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Table 6. Glucose concentration after enzymatic hydrolysis and the ethanol production from fermentation

Type & reaction time [min]	5	10	15	YPD	Raw cassava
Initial glucose conc. [g/L]	12.62	15.51	15.11	15.34	9.49
Max. ethanol conc. [g/L]	6.47	7.55	7.18	7.45	4.89
Ethanol yield [% of theoretical]	93.11	89.6	87.31	91.00	91.4

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