Optimization of the Enzymatic Saccharification Process of Empty Fruit Bunch Pretreated with Laccase Enzyme

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The saccharification of laccase-pretreated empty fruit bunch (EFB) was optimized in a lab-scale experiment using one-factor-at-a-time (OFAT) and response surface methodology (RSM). After pretreatment, the degree of delignification was checked by noting the weight loss (%) after pretreatment, and also by the quantity of total sugar produced after saccharification with cellulase enzyme. OFAT studies of saccharification of the pretreated EFB showed that the biomass was best saccharified using cellulase enzyme at the following conditions: enzyme concentration of 30 IU/g of EFB, substrate concentration of 5.0% w/v, 50 °C, saccharification time of 24 h, and pH 5. This combination exhibited the highest yield of total sugar (28% w/w). Although 29% w/w yield was achieved with an enzyme concentration of 40 IU/g of EFB, this increase in yield was not proportional to the increased enzyme concentration and, therefore, was considered insignificant. Statistical analysis of the combined effects of pH and temperature showed that pH had a more significant effect than the temperature on the saccharification process, based on a $P < 0.05$ significance level. The effect of pH on total sugar production was more significant than the temperature in both linear and quadratic functions. In sum, the saccharification of laccase-pretreated EFB should follow the optimized process conditions achieved in the current study.

Keywords: Empty fruit bunch; Laccase enzyme; Saccharification; Sugar

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INTRODUCTION

The production of sugar from lignocellulosic biomass requires delignification of the substrate (Venkatesh and Pradeep 2013). Cellulose and hemicellulose are the major sugars in empty fruit bunch (EFB), a residue in the palm oil industry. Lignocellulosic biomass is a preferred raw material for the production of bioethanol (Sudiyani *et al.* 2010). A major problem with the conversion of lignocellulosic biomass to bioethanol is the high percentage of lignin in cell walls, which protects cellulose and hemicellulose from cellulolytic enzymes (Havannavar and Geeta 2010). Different pretreatments have been employed to enhance the recovery of sugar from lignocellulosic biomass, with varying success in delignification. Chemical pretreatment with acids and bases is the method of choice because of its effectiveness (Iroba *et al.* 2013).

Although chemical pretreatment methods have been successfully adopted, they have many drawbacks including the formation of inhibitory factors such as furfural and hydroxymethyl furfural, low digestibility of produced sugar, and enzyme inhibition during hydrolysis (Sun and Cheng 2002). These factors contribute to the low yield of bioethanol from mostly chemically pretreated biomass. Thus, other ways of pretreating biomass for

the recovery of sugar and better ethanol yield during fermentation have been explored (Ukaegbu *et al*. 2014). Various factors that affect the successful saccharification of biomass include the nature and size of the biomass particles, the temperature of the saccharification, the pH of the medium, the concentration of substrate and enzymes, and the presence of inhibitory factors (Sanjeev *et al.* 2002, Shah *et al.* 2011, Sharma *et al.* 2013). To enhance the production of fermentable sugar from biomass during saccharification, it is common to pretreat the biomass with agents that have a lower tendency to produce inhibitory materials. Lignolytic agents, such as the laccase enzyme, have been useful in the fabrics and textile industries for pulping and softening materials, and their application in the fuel industry is being investigated (Galhaup *et al.* 2002).

In this study, laccase enzyme was used as a pretreatment agent for the delignification of EFB in a buffered solution. After delignification, the biomass was saccharified with cellulase enzyme for the production of sugar. The saccharification process parameters were optimized using OFAT and response surface methodology (RSM) to establish the best combination of parameters for maximum sugar recovery. Parameters screened in OFAT included the temperature of saccharification, pH, time, enzyme concentration, and substrate concentration. During the RSM studies, the pH and temperature of the saccharification were screened.

EXPERIMENTAL

EFB Collection

The EFB was collected from Dominion Square Sdn Bhd Oil Mill, Gambang Pahang, Malaysia. The sample was washed and dried until a constant weight was reached, after which the sample was milled to 2 mm size.

EFB Pretreatment

The EFB was pretreated with laccase enzyme 51003 from *Myceliophthora thermophilia*, supplied by Novozymes, Bagsværd, Denmark as described by Ukaegbu *et al.* (2016). During the pretreatment process, the degree of delignification was assessed by determining the percentage of the initial weight lost after pretreatment and also by determining the amount of total sugar produced after saccharification of the pretreated EFB. The pretreatment of the EFB was carried out in a reaction mixture made up of laccase enzyme concentration of 20 IU/g of EFB and EFB concentration of 5%w/v. Time of pretreatment was maintained for 4 h at 25 ^{o}C, in a citrate -phosphate buffer of pH 5, and agitation at 150 rpm.

Analytical Methods and Buffer preparation.

Determination of lignin, cellulose, hemicellulose and ash content of EFB

The sequential fractionation of EFB was carried out before and after pretreatment using a modified method described by Datta (1981). One gram of sample was suspended in 100 mL distilled water, kept at 100 °C for 2 h in a water bath, and filtered on a tared crucible. The residue was dried at 90 °C to constant weight. The loss was considered as the water soluble part. Two grams of dried EFB was suspended in 100 mL of 0.5 M H_2SO_4 and after keeping for 2 h at 100 °C in a water bath, the contents were filtered, dried, and weighed as described in the first step. Loss in weight was represented as hemicellulose content. For cellulose and lignin estimations, 10 mL of 72% (v/v) H_2SO_4 was added to the

above-dried residue and kept at 30 °C for 1 h on a rotary shaker at 200 rpm. After incubation, the mixture was diluted up to 4% (v/v) of H_2SO_4 and autoclaved at 1.06 kg/cm^2 for 40 min. The contents were filtered, dried, and weighed. The loss in weight was treated as cellulose, and the leftover residue was considered as lignin. For estimating the residual ash content, 1 g of sample was kept at 550 ºC for 5 h in a tared crucible and reweighed to calculate the residual ash content.

Determination of total sugar content

The determination of total sugar after saccharification was carried out using a phenol-sulfuric acid method described by Dubois *et al.* (1956). In the modified method, 100 µL of sample filtrate was added to a glass tube, followed by the addition of 50 µL of 80% phenol (w/v). The tubes were vortexed for 30 s, before 2 mL of 98% concentrated sulphuric acid was added in a stream and vortexed for a second time. The tubes were allowed to stand for 10 min at room temperature. The absorbance of the developed color was read at 490 nm using a microplate reader model Infinite pro-TECAN. The concentration of total sugar in the sample was read from an already prepared standard graph.

Determination of reducing sugar content

The reducing sugar content of the saccharified EFB was determined using the dinitro salicylic acid (DNS) method described by Miller (1959). Centrifuged sample filtrate (1.5 mL) was added into a 25 mL glass test tube with screw cap, followed by the addition of 3 mL of DNS reagent into the tube. The tube was placed in a boiling water bath for 5 min, which after cooling, 10 mL of distilled water was added into the tube and the content of the tube was vortexed to homogeneity. The absorbance of the developed color was read at 540 nm in a microplate reader model Infinite pro-TECAN and the concentration of reducing sugar in the sample was read from an already prepared standard graph.

Preparation of 50 mM sodium acetate buffer

Cellulase enzyme used in the saccharification study was prepared having a concentration of 50 mM by dissolving 6.8 g of sodium acetate trihydrate in one liter of distilled water, in a volumetric flask. Furthermore, 2.87 mL of glacial acetic acid was added to the flask, and the final volume was made up to one liter. The resulting pH of the buffer was adjusted to the required pH with either 0.5M sodium hydroxide solution or 0.5M sulphuric acid solution, depending on the required pH.

Determination of laccase enzyme activity

The laccase enzyme activity of the Novozym 51003 was determined using the 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) method in a 0.1 M sodium acetate buffer of pH 4.5. The ABTS-buffer solution was prepared with 0.1 M sodium acetate buffer pH 4.5, and 0.4 mM ABTS at 25 ºC. The reaction mixture contained 0.58 mL of the ABTS-buffer solution and 0.02 mL of the Novozymes enzyme in a total volume of 0.6 mL. One unit of the enzyme was defined as the amount of the laccase enzyme that will oxidize 1 µmol of ABTS per minute.

Determination of cellulase enzyme activity

Cellulase enzyme used in the study was purchased in powder form and the activity was determined by using standard filter paper (1.0 x 6.0 cm) incubated with the enzyme at 50 ºC for 1 h in a sodium acetate buffer of pH 5.0 prepared as described in Appendix C. The reducing sugar released by the enzyme was estimated using the DNS method described in section 3.3.3. One unit of the enzyme was defined as the amount of enzyme that will liberate one micromole of reducing sugar per minute and the unit was expressed in international units (IU). The enzyme was reconstituted in de-ionized water to a concentration of 1 IU/µL before use.

Optimization of the Saccharification Process Parameters using OFAT

Enzyme concentration

Five enzyme concentrations of 5, 10, 20, 30, and 40 IU/g of EFB were studied. The process condition was made up of: time 24 h, temperature 50 ºC, pH 5, and EFB concentrations of 5% w/v. All the experiments were carried out in triplicates and the results were presented as the mean of the triplicates.

EFB concentration

Four EFB concentrations of 5, 10, 15, and 20% w/v were studied. The process condition was made up of enzyme concentration 5 IU/g of EFB, time 24 h, temperature 50 ºC, and pH 5. The enzyme concentration was reduced to 5 IU/g to minimize the consumption of the enzyme during the study.

pH

The effect of the pH of the saccharification process was studied using buffer pH range of 3, 4, 5, 6, and 7. The process condition was the same with the condition during the study of the EFB concentration, only the buffer pH was varied.

Duration (Time)

The effect of time on the saccharification of the EFB was studied for the durations of 12, 24, 36, and 48 h. The process condition was the same with the condition during the study of the EFB concentration, except that the duration of the process was varied.

Temperature

The effect of the temperature of saccharification process was studied at four different temperatures of 40, 50, 60, and 70 °C. The process condition was the same with the condition during the study of the EFB concentration, only the temperature of the process was varied.

Optimization of Saccharification Parameters using RSM

The optimization of the saccharification of the enzyme-pretreated EFB using cellulase enzyme was studied in a statistical model. Design-Expert version 6.0.8 was used to design the experiments, adopting the Face Centered Central Composite Design (FCCCD). The FCCCD was chosen over other methods of RSM optimization because it gives a more defined boundary without the need for the introduction of values that are not obtainable around the defined points. Two process parameters (pH and temperature) were studied. The enzyme concentration, substrate concentration, and the time were maintained at 5 IU/g of EFB, 5% w/v, and 24 h, respectively. The responses were presented in mg/mL.

All experiments were carried out in triplicates, and the results were presented as the mean of the triplicates.

Validation of the Developed Model

After the optimization of the saccharification process using RSM, the developed model was validated by conducting five experimental set-ups suggested by the model. The temperatures during the validation process were 48.02, 45.80, 50.20, 31.12, and 50.96 ºC; while the suggested pH values were 5.71, 6.16, 6.96, 3.65, and 4.33. All the experiments were done in triplicates, and the results were presented as the mean of the triplicates. Note that only the developed model for the total sugar prediction was validated.

RESULTS AND DISCUSSION

EFB Characterization

The results of the EFB sequential characterization of EFB before pretreatment and after pretreatment at the OFAT determined process conditions using laccase enzyme is shown in Table 1.

Table 1. Composition of Characterized EFB (%)

EFB condition	Cellulose	Hemicellulose	Lignin	Ash
Un-pretreated (control)	38.7	32.2	23.3	5.8
Pretreated at optimized condition	42.1	34.7	18.6	4.0
Percentage lignin loss			20.1	

n = 3

Lignocellulose biomass consists mainly of cellulose and hemicellulose and an appreciable amount of lignin which intertwined with the sugar molecules to provide strength and shield. The approximate percentage composition of each composition varies depending on the source of the EFB as reported by Alvira *et al.* (2010). The result was found to be in agreement with the findings, which reported that EFB contains more cellulose (49.6%) than hemicellulose (21.2%), and also 18% of lignin and 2% of ash as well though EFB collected from different environments may differ in the percentage composition of these components.

Effect of Enzyme concentration

The effect of the cellulase enzyme concentration on the rate of enzymatic saccharification of the laccase enzyme-pretreated EFB was studied using the enzyme concentrations of 5, 10, 20, 30, and 40 IU/g of EFB. There was a progressive increase in saccharification rate with increasing enzyme concentration until the point of enzyme saturation (Fig. 1). There was little or no effect on the rate of saccharification when the enzyme concentration was further increased after the saturation point. At a cellulase enzyme concentration of 5.0 IU/g of EFB, the total sugar yield was 4.0% (w/w) after 24 h of saccharification at 50 °C. When the concentration was increased to 10 IU/g of EFB, the yield increased to 7.0% (w/w). At 20 and 30 IU/g of EFB, the yield of total sugar was 14% and 28% (w/w), respectively. At this point, further increasing of the cellulase enzyme concentration to 40 IU/g of EFB yielded only a minimal increase in the total sugar (29%

w/w). The production of reducing sugar was also increasing with the increase in the enzyme concentration in a similar way as total sugar yield. These results were expected because of enzyme saturation kinetics.

These results were consistent with published data. Phuengjayaem *et al.* (2014) studied the saccharification of sweet sorghum with cellulase enzyme at different concentrations using RSM. A total of 0.058 g and 0.139 g of glucose/gram of dry substrate was recovered with an enzyme concentration of 20 FPU/g and 30 FPU/g of glucose/gram of dry substrate, respectively, showing an increase in the saccharification rate with increasing enzyme concentration. Also, Jagatee *et al.* (2015) optimized the saccharification of sweet potato for maximum ethanol recovery using two hydrolytic enzyme combinations. They showed increased saccharification when the enzyme concentration was increased from 15 to 20 µL, resulting in a corresponding increase from 100 to 400 mg/g of total sugar. A decline in the total sugar (< 400 mg/g) concentration was reported when the enzyme concentration was increased from 21 to 25 µL. The enzyme concentration for other process parameters was reduced to 5.0 IU/g of EFB to limit the consumption of enzyme during the study.

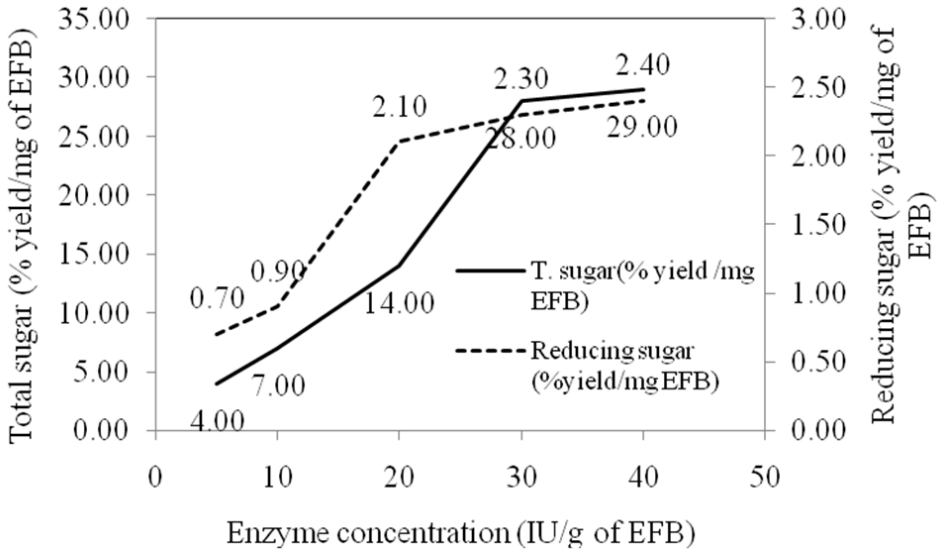


Fig. 1. Effect of enzyme concentration on the rate of saccharification of enzyme-pretreated EFB

Effect of Substrate Concentration

The effect of substrate concentration on the rate of enzymatic saccharification of the enzyme-pretreated EFB is shown in Fig. 2. The results showed a marked decrease in the rate of total sugar and reducing sugar yields as the substrate (EFB) concentration was increased. A substrate concentration of 5.0% w/v had the highest yield of total sugar (4.0% w/w), and reducing sugar (0.72% w/w) compared with 1.0% w/w of total sugar and 0.02% w/w of reducing sugar produced when the substrate concentration was increased to 15% w/w.

Compared with other studies that optimized the substrate concentration of saccharification, this result was similar. Sirous *et al.* (2013) optimized enzymatic saccharification of lignocellulosic materials at different solid: liquid ratios and obtained a maximum sugar concentration of 261 ± 7.9 mg/g of the substrate at a 1:10 w/v ratio. When the ratio was increased, the concentration of sugar was reduced. Phuengjayaem *et al.*

(2014) also studied the effect of substrate concentration of sorghum on its enzymatic saccharification using RSM and recorded a higher saccharification response (0.069 g/g of dry substrate) with a lower substrate concentration of 2.5% w/v, compared with 0.017 g/g obtained with a substrate concentration of 5.5% w/v. These results confirmed that increasing the substrate concentration of the enzymatic saccharification of biomass leads to slower saccharification because of an increased consistency and reduced surface contact between enzyme and substrate.

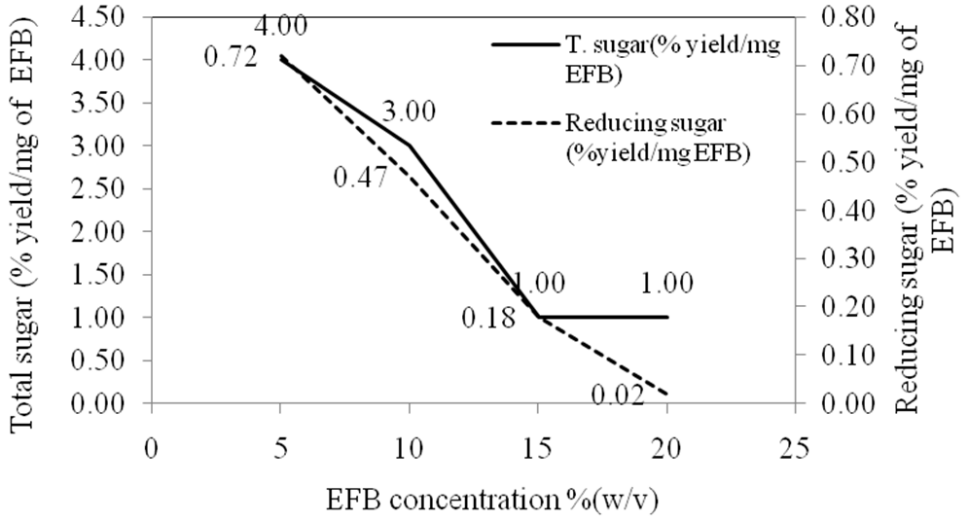


Fig. 2. Effect of the substrate concentration on the rate of saccharification of enzyme-pretreated EFB

Effect of Medium pH

pH was a major factor in determining the rate of enzymatic saccharification of enzyme-pretreated EFB (Fig. 3).

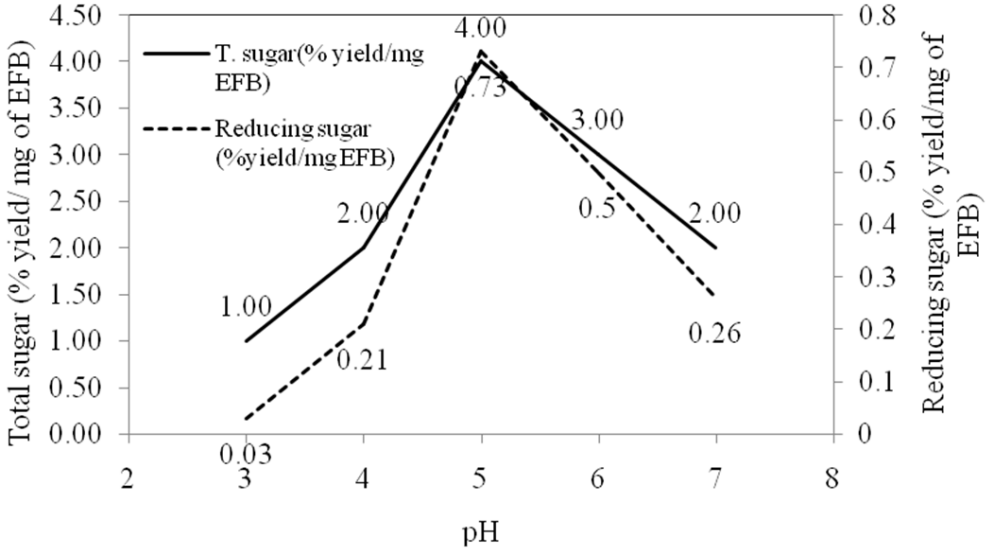


Fig. 3. Effect of medium pH on the rate of saccharification of enzyme-pretreated EFB

While all enzymes have a specific range of pH, most hydrolytic enzymes work better at a pH range of 3 and 6. The maximum total sugar (4.0% w/w) and reducing sugar (0.73% w/w) were produced when the pH was 5, and the minimum yields of 2.0% w/w and 0.03% w/w respectively were produced when the pH was 7. Thus, the best performance for the cellulase enzyme was at pH 5, and a further increase or decrease in the pH led to a sharp decline in the yields. These results indicated that the isoelectric point of the enzyme reaction was reached at pH 5 when the rate of saccharification was maximal. Phuengjayaem *et al.* (2014) studied the effect of pH of the medium on the saccharification of sweet sorghum using RSM. They discovered that 0.115 g of glucose /g of dried solid were obtained at a pH of 5, compared with 0.00 g/g obtained when the pH was 7, despite the model prediction of 0.026 g/g of dried solid. These results confirmed that the pH of the medium is very important for controlling the rate of biomass saccharification using enzymes.

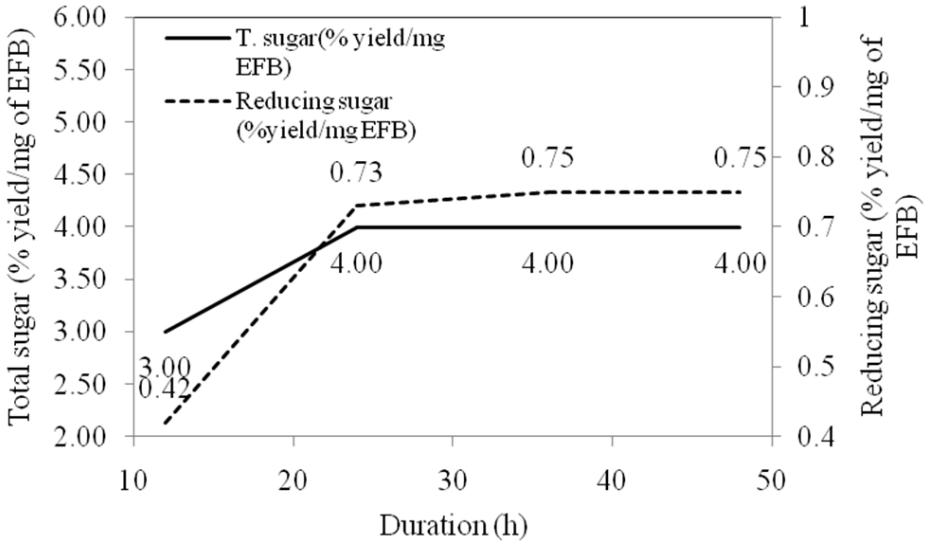


Fig. 4. Effect of time on the rate of saccharification of enzyme-pretreated EFB

Effect of Duration (Time)

Results of the effect of reaction duration on the enzymatic saccharification of the pretreated EFB showed that the reaction time more than 24 h contributed little to the rate of saccharification. The results shown in Fig. 4 indicated that after 24 h, the rate of sugar production tended to be static even with the increase in time. The rate of saccharification can be increased with time only when enzymes are still effectively engaged with the biomass. In the absence of more active enzymes, longer time contributes little or nothing to the rate of sugar production. After 12 h of saccharification, the percentage of total sugar and reducing sugar yields were 3.0% (w/w), and 0.42% (w/w), respectively. After 24 h, the total and reducing sugar yields increased to 4.0% and 0.73% (w/w), respectively. When prolonged to 36 and 48 h, the yields of total sugar remained stable at 4% (w/w) while reducing sugar had a little increase from 0.73 to 0.75% (w/w). This showed that production of total sugar gets to maximum after 24 h of saccharification. Sirous *et al.* (2013) studied the effect of saccharification reaction time up to 96 h during optimization of saccharification condition of water hyacinth. They obtained the maximum sugar yield of 290 mg/g of biomass after 48 h of saccharification and reported a decrease when prolonged

to 96 h. Zhu *et al.* (2008) also reported that the rate of hydrolysis of biomass depends on many factors including the time, concentration of enzyme and substrate and also on the structural features of the biomass resulting from the type of agent used during pretreatment. This has been demonstrated in this work that saccharification of EFB with cellulase enzyme can be achieved within 24 h hence reducing the time needed for enzymatic saccharification of EFB by 50%.

Effect of Temperature

Results of the effect of temperature on the rate of enzymatic saccharification of enzyme pretreated EFB are shown in Fig. 5. From the results, it was observed that the optimum temperature for the enzymatic saccharification of the enzyme pretreated EFB was 50 °C. Saccharification at temperatures lower or higher than 50 °C showed reductions in the saccharification responses. At 30 °C and 40 °C, the percentage yields of total and reducing sugar were 2.0%, 0.18%, and 2.0%, 0.31%, respectively. When saccharification was done at 50 °C, the total and reducing sugar yields were 4.0% and 0.73%, respectively, which were the highest yields observed. When the temperature was increased to 60 and 70 °C, the total and reducing sugar yields were reduced to 3% and 2%; and 0.61 and 0.13%, respectively. This was believed to be due to the progressive thermal denaturation of the enzymes at 60 and 70 °C.

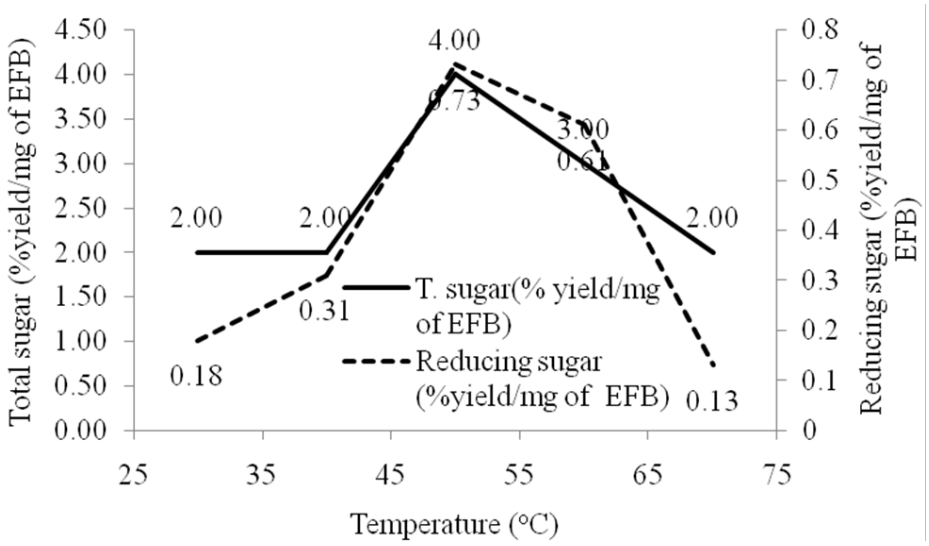


Fig. 5. Effect of temperature on the rate of saccharification of enzyme-pretreated EFB

Studies previously conducted on the effect of temperature on the rate of enzymatic reactions confirmed that when the temperature was higher than the tolerance limit of an enzyme system, the protein structures that maintain the shape and function of the enzyme will become denatured, and this will result in the loss of the enzyme activity (Martinek 1969). Pandiyan *et al.* (2014) optimized the enzymatic saccharification of alkali-pretreated *Parthenium* spp. using response surface methodology and achieved 83.27% saccharification efficiency at 50 °C, which was higher than 22.16% saccharification efficiency recorded at 60 °C. These were indications that the temperature of 50 °C was the most favored temperature for the enzymatic saccharification of different lignocellulosic biomass. Sun and Cheng, (2002) also noted that saccharification of lignocellulosic materials using hydrolytic enzymes performs better under mild conditions (pH 4.8 and

temperature 50 °C), which are more favorable to the enzymes. Considering these reports and findings, the results of this study are believed to be an expected outcome.

Effect of Experimental Factors (pH and Temperature)

Enzyme catalyzed reactions are dependent on the external environment, including the temperature and pH.

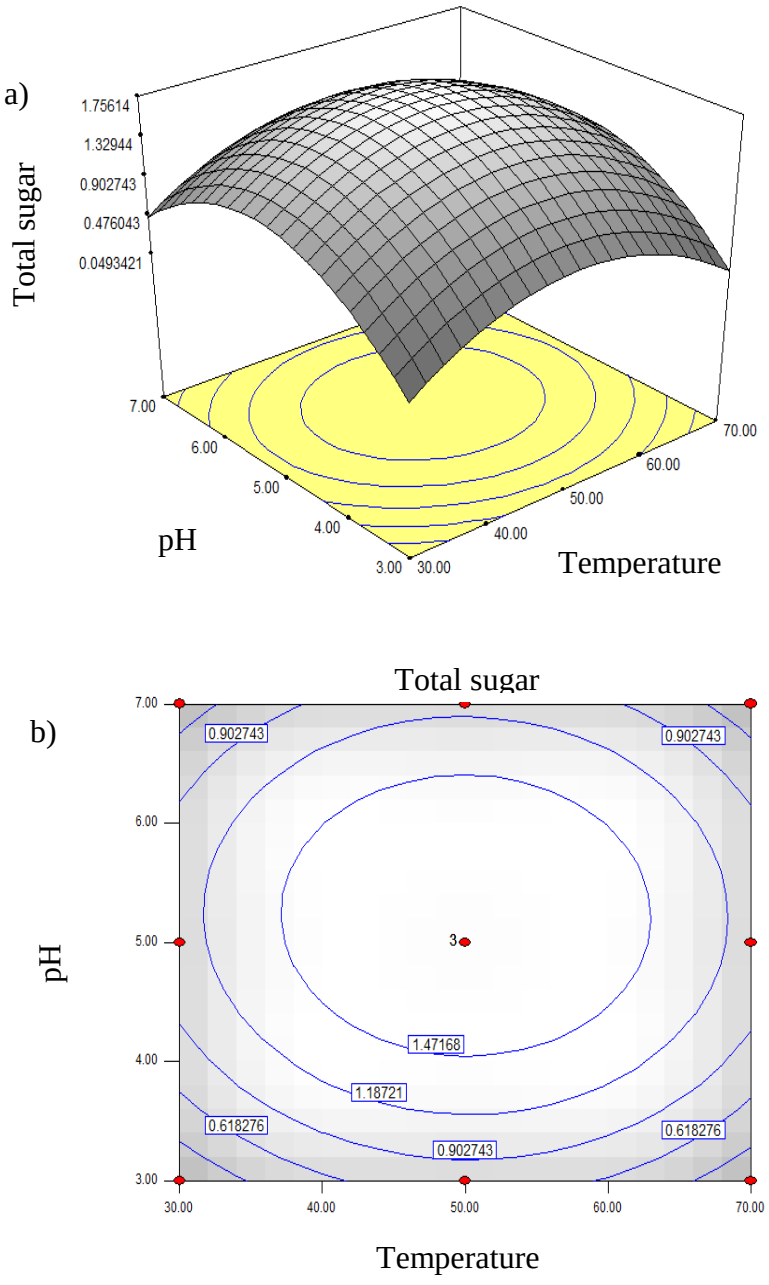


Fig. 6. The a) three-dimensional response and b) contour plot of the effects of temperature and pH on the total sugar yield

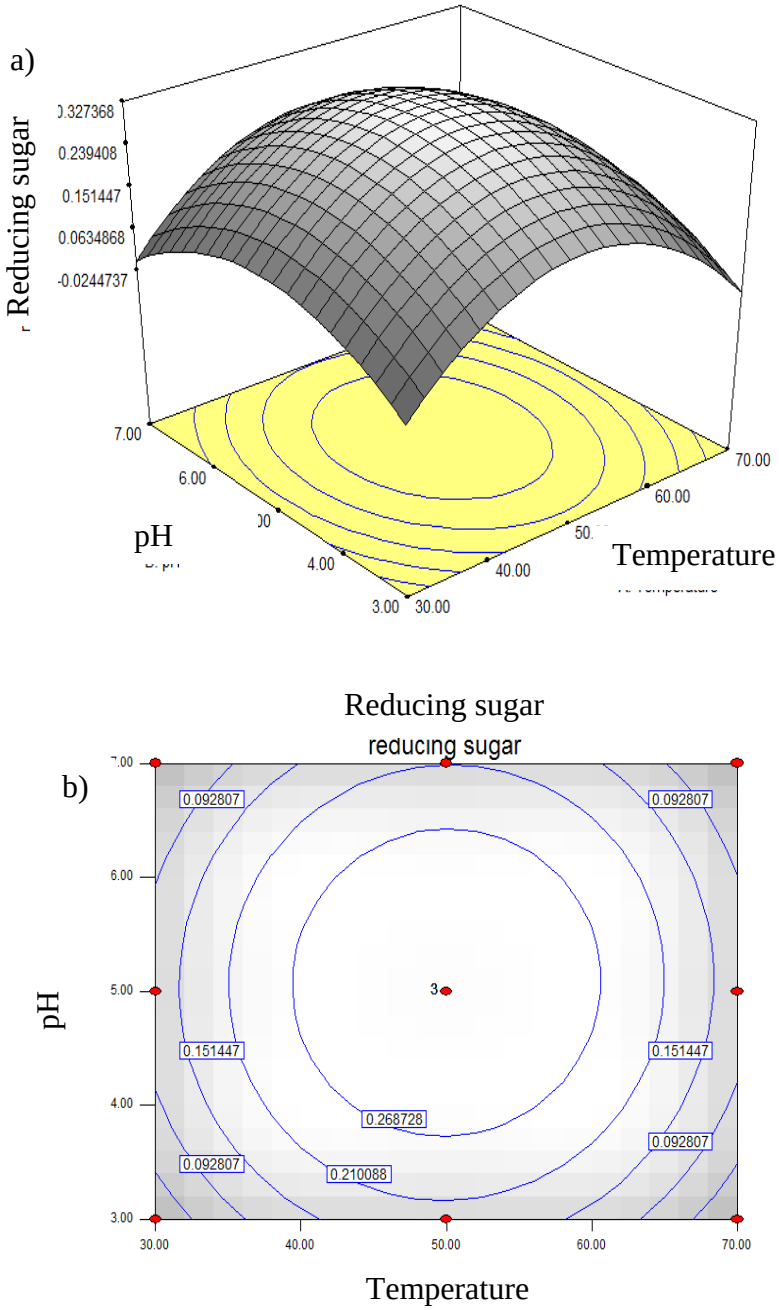


Fig. 7. The a) three-dimensional response and b) contour plot of the effects of temperature and pH on the reducing sugar yield

Often, hydrolytic enzymes are active within the pH range of 3 to 6, and their activity is influenced by the way the enzyme binds to the substrate, the substrate ionization, and the protein structure variation at extreme pH (Bayındırlı 2010). In this study, the effect of temperature and pH on the saccharification of the enzyme-pretreated EFB using cellulase enzyme was studied. The 3-D response and contour plots showing the effects of both temperature and pH on the yields of total sugar and reducing sugar are depicted in Fig. 6 (a and b) and Fig. 7 (a and b).

The semi-spherical shapes of the 3-D response plots displayed increasing total sugar and reducing sugar with increases in temperature and pH towards 50 °C and pH 5, respectively. These increases were maintained until the point of enzyme thermal denaturation, which resulted in declined in response at a temperature above 50 °C and pH above 5. These increasing trends were expected because cellulase activity is highly influenced by temperature and pH, ranging from 45 to 60 °C, and 3 to 6, respectively. The total sugar increased from 0.18 mg/mL at 30 °C and pH 3 to a maximum of 1.8 mg/mL at 50 °C and pH 5, while reducing sugar increased from 0.01 mg/mL to 0.36 mg/mL at the same change in temperature. Different cellulase enzymes vary in their tolerance to pH and temperature. However, the observations from this study are consistent with the findings of Pandiyan *et al.* (2014), who studied the combined effects of pH and temperature on the enzymatic saccharification of alkali-pretreated *Parthenium* spp. using response surface methodology. These authors achieved 83.27% saccharification efficiency at 50 °C and pH 5, which was higher than the 22.16% saccharification efficiency recorded at the temperature of 60 °C and pH 6. Phuengjayaem *et al.* (2014) studied the effect of pH of the medium on the saccharification of sweet sorghum using RSM and found that at pH 5, 0.115 g/g of dried solid was obtained, compared with 0.00 g/g obtained at pH 7, even though the model predicted 0.026 g/g of dried solid.

The contour plots in Fig. 6b and 7b show the interaction effect of temperature and pH on the rate of saccharification. The pH and temperature interacted around the central points in an almost similar distribution, showing that the process was optimized, although the effect of pH was more pronounced compared with the effect of temperature. The predicted and experimental yields of total sugar and reducing calculated as the mean of the triplicates are presented in Table 2 and 3.

Table 2. Predicted and Experimental Total Sugar Concentrations

Standard	Run	Block	Temperature (°C)	pH	Total Sugar (mg/mL)		
					Experimental	Predicted	% Error
10	1	1	50	5	1.80	1.75	3.00
3	2	2	30	7	0.41	0.45	-10.80
5	3	2	30	5	0.95	1.07	-12.20
2	4	2	70	3	0.21	0.09	55.10
11	5	1	50	5	1.78	1.75	1.90
9	6	1	50	5	1.80	1.75	3.00
8	7	2	50	7	1.21	1.12	7.70
1	8	2	30	3	0.18	0.05	72.60
7	9	2	50	3	0.50	0.75	-49.30
6	10	2	70	5	1.00	1.08	-7.60
4	11	2	70	7	0.39	0.43	-10.10

Total sugar yield (Table 2) increased from 0.18 mg/mL when saccharification was done at 30 ºC and pH 3 to a maximum of 1.8 mg/mL when performed at 50 ºC and pH 5. The results of this study showed that at a temperature higher than 50 ºC, denaturation of the protein structure of the cellulase enzyme may occur, leading to a loss of enzyme activity and reduction in the conversion of the substrate to product. Production of reducing sugar was also affected by both factors as seen in Table 3. Reducing sugar production by the enzymes also showed an increasing trend as the temperature and pH were varied,

increasing from 0.01 mg/mL when saccharification was done at 30 °C and pH 5 to 0.36 mg/mL when saccharification was done at 50 °C and pH 5.

Table 3. Predicted and Experimental Reducing Sugar Concentrations

Standard	Run	Block	Temperature (°C)	pH	Reducing Sugar (mg/mL)		
					Experimental	Predicted	% Error
10	1	1	50	5	0.36	0.33	8.30
3	2	2	30	7	0.01	0.00	100.00
5	3	2	30	5	0.07	0.12	-71.40
2	4	2	70	3	0.01	-0.02	300.00
11	5	1	50	5	0.36	0.33	8.30
9	6	1	50	5	0.36	0.33	8.30
8	7	2	50	7	0.17	0.21	-23.50
1	8	2	30	3	0.01	-0.01	200.00
7	9	2	50	3	0.13	0.19	-46.20
6	10	2	70	5	0.06	0.12	-100.00
4	11	2	70	7	0.03	0.01	66.70

The analysis of variance (ANOVA) of the FCCCD for total sugar and reducing sugar are presented in Tables 4 and 5. The pH had a more significant effect on the responses (P = 0.0348) than the temperature (P = 0.9411)

Table 4. Analysis of Variance for Total Sugar

Source	Sum of Squares	DF	Mean Square	F-Value	P-value
Model	4.0556	5	0.8111	32.6467	0.0008
Temperature	0.0002	1	0.0002	0.0060	0.9411
pH	0.2054	1	0.2054	8.2651	0.0348
Temperature^2	1.1525	1	1.1525	46.3846	0.0010
pH^2	1.6805	1	1.6805	67.6392	0.0004
Temperature*pH	0.0012	1	0.0012	0.0493	0.8331
Residual	0.1242	5	0.0248	-	-
Lack of Fit	0.1242	3	0.0414	-	-
Pure Error	0.0000	2	0.0000	-	-
Corrected Total	4.1799	10	-	-	-

Table 5. Analysis of Variance for Reducing Sugar

Source	Sum of Squares	DF	Mean Square	F-Value	P-value
Model	0.2031	5	0.0406	12.8747	0.007
Temperature	0.0000	1	0.0000	0.0000	1.0000
pH	0.0006	1	0.0006	0.1920	0.6809
Temperature^2	0.1100	1	0.1100	34.8829	0.002
pH^2	0.0418	1	0.0418	13.2435	0.0149
Temperature*pH	1E-04	1	1E-04	0.0317	0.8657
Residual	0.0158	5	0.0032	-	-
Lack of Fit	0.0158	3	0.0053	-	-
Pure Error	0.0000	2	0.0000	-	
Corrected Total	0.2189	10	-	-	-

The total sugar yield (TSY) and reducing sugar yield (RSY) was represented by a polynomial equation, where *T* is the temperature of the reaction.

$$\text{TSY} = -8.14451 + 0.17107 * T + 2.15056 * pH - 1.6861E^{-003} * T^2 - 0.20362 * pH^2 - 4.37500 - 004 * TpH \quad (1)$$

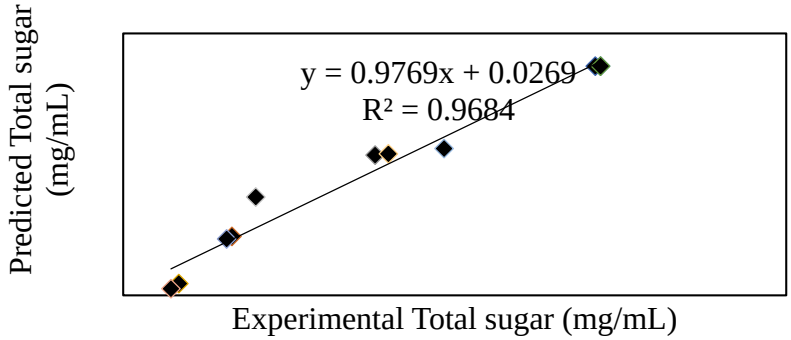
$$\text{RSY} = -1.77164 + 0.051480 * T + 0.31980 * pH - 5.21053E^{-004} * T^2 - 0.032105 * \quad pH^2 + 1.25000E^{004} * TpH \quad (2)$$

Based on the *P*-value of 0.0008, the model parameters obtained from RSM optimization using FCCCD were significant. The coefficient of determination (R^2) value of 0.9700 and 0.9279 for total sugar and reducing sugar, respectively obtained from the models, implied a strong correlation between the factors (temperature and pH) and the responses (Table 6).

Table 6. Analysis of Variance Parameters of the Model Fitted for Total Sugar and Reducing Sugar

Term	Total Sugar	Reducing sugar
P-value	0.0008	0.0070
F value	32.6400	12.8747
Mean	0.9300	0.1400
R^2	0.9700	0.9279

a)



b)

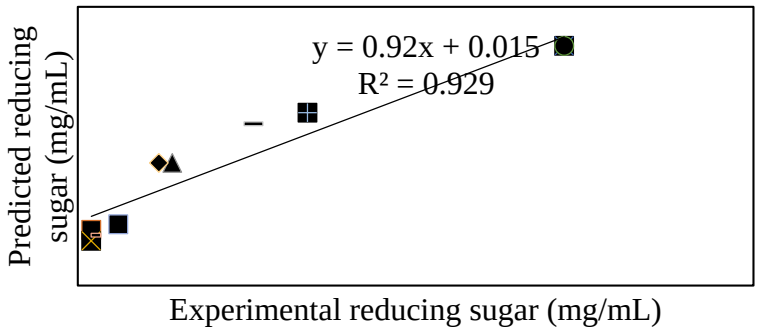


Fig. 8. Parity plots of the experimental and predicted values for the effects of temperature and pH on total and reducing sugar production

The parity plots of the experimental and the predicted values of total and reducing sugars are shown in Fig. 8 (a and b). The plots show that the model had a good predictability of the total sugar and reducing sugar correlated by the respective R^2 of 0.968 and 0.929, respectively.

Validation of the Developed Model

Five sets of experiments were carried out using the solutions from the model to validate the predictability of total sugar production. The experimental and predicted responses, as well as the percentage errors of the five selected solutions, are presented as the mean of the triplicates in Table 7. The mean error between the experimental and predicted yield of total sugar from the enzyme-pretreated EFB after saccharification with cellulase enzyme was 4.16%. Thus, the FCCCD model was statistically reliable up to 95.8% confidence for the prediction of the total sugar yield after saccharification.

Table 7. Validation of the Developed Model

Solution	Temperature (°C)	pH	Experimental (mg/mL)	Predicted (mg/mL)	% Error
1	48.02	5.71	1.67	1.69	-1.2
2	45.85	6.16	1.58	1.54	2.5
3	50.20	6.96	1.20	1.13	5.8
4	31.12	3.65	0.68	0.63	7.4
5	50.96	4.33	1.52	1.58	-3.9

CONCLUSIONS

1. The optimized process conditions were an enzyme concentration of 30 IU/g of EFB, substrate concentration of 5.0% w/v, the temperature of 50 °C, pH 5, and duration of 24 h. pH had a significant effect on the saccharification process.
2. The optimal process parameters obtained here agreed with previous studies; however, a shortened time of saccharification was optimized at 24 h. This result was attributed to the absence of inhibitory substances in the saccharification mixture.
3. The pH must be considered for effective saccharification of enzyme pretreated EFB.

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