CHAPTER 4

RESULTS & DISCUSSIONS

4.1 Preliminary Studies: Selection of the Most Effective Chemical in Banana Trunk Biomass Pretreatment

Table 4.1 shows the preliminary study of the glucose yield in the acidic and alkaline solutions. From the findings, 2% (v/v) H_2SO_4 acidic solution (glucose yields = 64.67 g/L) was the most effective chemical for pretreatment of banana trunk biomass compared to 2% (w/v) NaOH alkaline solution (glucose yields = 51.10 g/L) under constant pretreatment conditions of 10% (w/v) substrate concentration pretreated exactly 30 minutes at 100 ℃. The higher glucose formation from acidic pretreated biomass indicated that acid pretreatment relatively suitable for banana trunk as low lignin content biomass. This justification was agreed by the studies of Harmsen *et al*. (2010) as stated in Table 4.2 which shows the comparison of action mode between acidic and alkaline pretreatment on lignocellulosic biomass. The lignocellulosic properties of banana trunk with 5 – 10% lignin content only also been proved by the paper of Preethi and Balakrishna (2013). The high suitability of acid pretreatment for low lignin content biomass can be explained that acids are powerful agents for hemicellulose and cellulose hydrolysis which enhance the porosity and improve the enzymatic digestibility of biomass without the protective layer of lignin as mass transfer barriers of acids to biomass.

Table 4.1: Comparison studies between 2% (v/v) H_2SO_4 solution (acidic) and 2% (v/w) NaOH solution (alkaline) in banana trunk biomass pretreatment.

Chemical type	Glucose concentration (g/L)
2% (w/v) NaOH	51.10
2% (v/v) H_2SO_4	64.67

Note: This experimental activity was conducted in triplicates with analysis of standard deviation (refer Appendix D)

Table 4.2: Mode of action comparison between acidic and alkaline pretreatment on lignocellulosic biomass (Harmsen *et al*., 2010).

Type of pretreatment	Mode of action
Acidic pretreatment	• Major in removing hemicellulose • Minor in altering lignin structure • Promote hydrolysis of cellulose and hemicellulose • Highly suitable for low lignin content biomass
Alkaline pretreatment	• Major in removing lignin • Minor in removing hemicellulose • Highly suitable for high lignin content biomass

Acidic pretreatment of 2% (v/v) H_2SO_4 was selected for further pretreatment conditions optimization studies on the effect of substrate concentration (% (w/v)), treatment duration (min), and treatment temperature (℃) according to its higher glucose production in subsequent enzymatic hydrolysis compared to alkaline pretreatment with same concentration of 2% (w/v) NaOH.

4.2 One-Factor-at-A-Time (OFAT) Studies

Minimization of the bias impact on the experimental parameter results was done by preventing any randomization of factorial design and according to the research of literature review. Three critical parameters of substrate concentration , treatment duration, and treatment temperature were studied in the range of 4% (w/v) – 10% (w/v), 10 minutes – 40 minutes, and 40 ℃ – 100 ℃ respectively.

4.2.1 Effect of Substrate Concentration

Figure 4.1 shows substrate concentration of 10% (w/v) contributes the highest glucose concentration (64.67 g/L) in subsequent enzymatic hydrolysis after pretreatment under constant conditions of 30 minutes pretreatment at 100 ℃ among substrate concentration of 4% (w/v), 6% (w/v), and 8% (w/v). The increasing trend of substrate concentration in subsequent glucose yields indicates that higher glucose formation can be obtained for 10% (w/v) of substrate concentration in biomass pretreatment. This hypothesis can be proved by the review article made by Harmsen *et al*. (2010) in which stated that high porosity formation of pretreated substrate can be obtained under high substrate concentration (10 – 40% (w/v)) for temperature lower than 160 ℃.

The studies of Harmsen *et al*. (2010) showed the pretreated wheat straw with dilute sulphuric acid under research condition of 20% (w/v) of substrate concentration at 150 ℃ offered good performance in term of sugar production in hydrolysis process. This can be justified that sufficient supply of substrate supply can increase the biomass surface availability for chemical to disrupt the lignocellulosic content by creating pores on the substrate in order to release high amount of cellulose for further glucose conversion in subsequent enzymatic hydrolysis step.

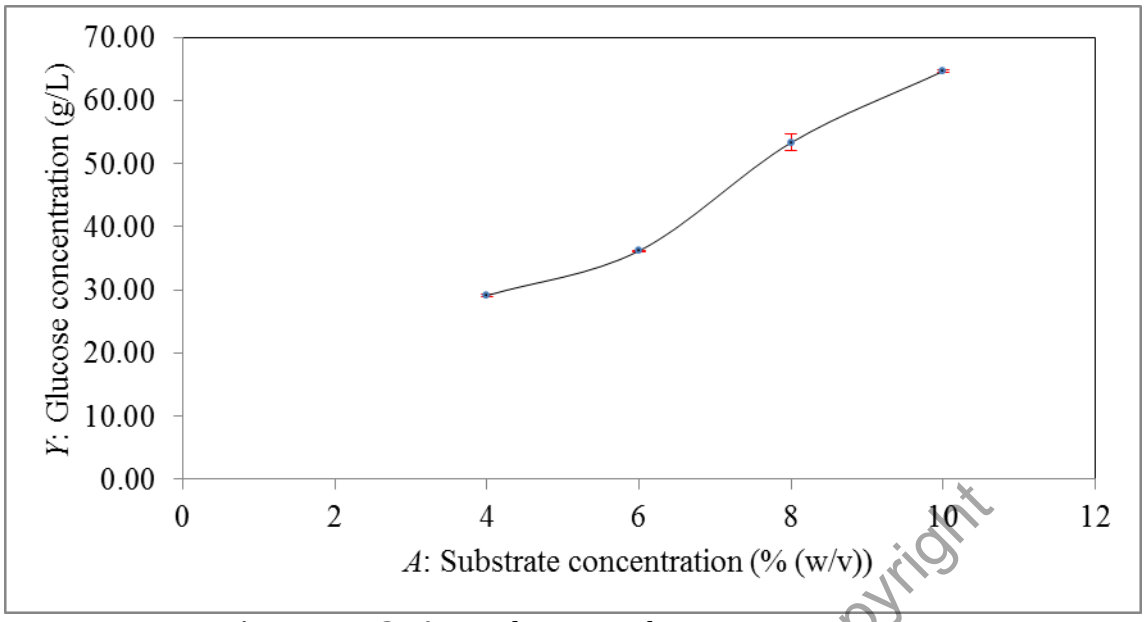

Figure 4.1: OFAT analysis on substrate concentration.

4.2.2 Effect of Treatment Duration

Figure 4.2 shows treatment duration of 30 minutes contributes the highest glucose concentration (64.67 g/L) in subsequent enzymatic hydrolysis after pretreatment under constant conditions of 10% (w/v) substrate concentration immersed in 100 ℃ water bath among treatment concentration of 10 minutes, 20 minutes, and 40 minutes. The glucose yields drop after 30 minutes of pretreatment can be explained by the reason of enzyme inhibitors formation (furfurals and HMF) which can influence the efficiency of subsequent enzymatic hydrolysis in glucose production. This hypothesis is supported by the statement in papers by Singh and Trivedi (2013) and Chidi *et al*. (2015) in which formation of inhibitors most probably can be occur above 30 minutes of biomass pretreatment below the temperature of 120 ℃.

The inhibitors have toxic effects on the fermenting organisms by affecting the cell growth and respiration. Kinetic studies have shown that the production of inhibitors strongly increases with residence time of acid pretreatment (Harmsen *et al*., 2010). Therefore, the optimal approach is to prevent the formation of inhibitors as much as possible through the pretreatment process conditions.

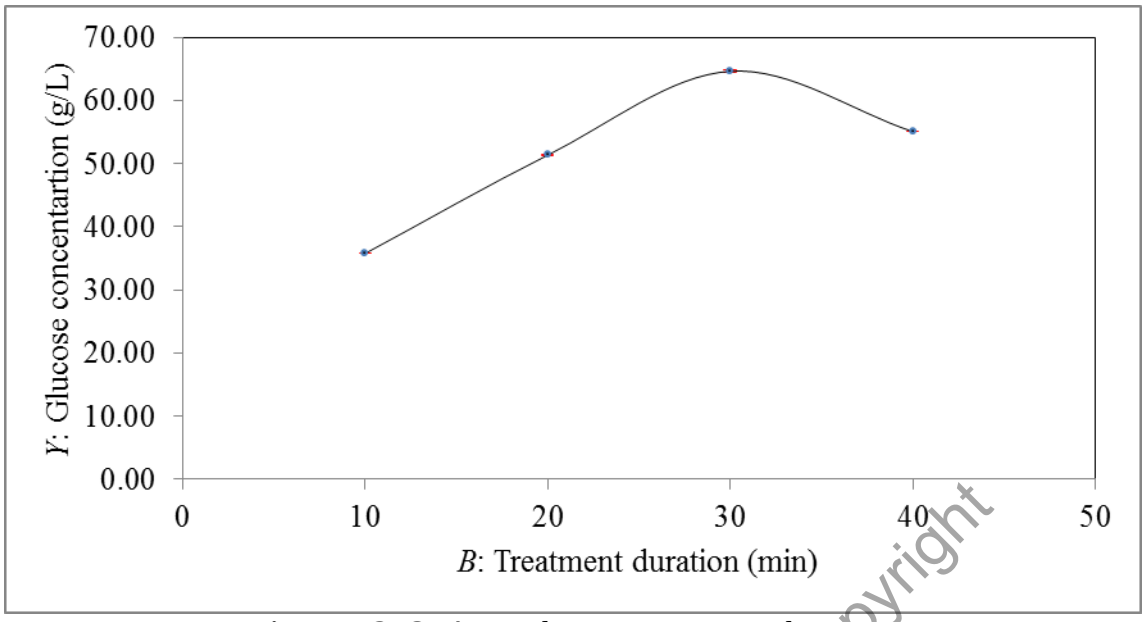

Figure 4.2: OFAT analysis on treatment duration.

4.2.3 Effect of Treatment Temperature

Figure 4.3 shows treatment temperature of 100 ℃ contributes the highest glucose concentration (64.67 g/L) in subsequent enzymatic hydrolysis after pretreatment under constant conditions of 10% (w/v) substrate concentration pretreated for period of 30 minutes among treatment temperature of 40 ℃, 60 ℃, and 80 ℃. The increasing trend of treatment temperature in subsequent glucose yields indicates that higher glucose formation can be obtained after 100 ℃ of treatment temperature in biomass pretreatment. This hypothesis can be explained according to the report by Myat and Ryu (2015) that stated optimum biomass pretreatment temperature most probably can be highly achieve until approximately 160 ℃ without formation of enzyme inhibitors.

Although high temperature is essential to provide heating energy for the process of modification of lignin structure and depolymerisation of hemicellulose; however, the increase of temperature without controlling and restriction will lead to the further degradation of sugar (glucose) to furfural and HMF (enzyme inhibitors) via chemical processes of isomerization and dehydration (Harmsen *et al*., 2010).

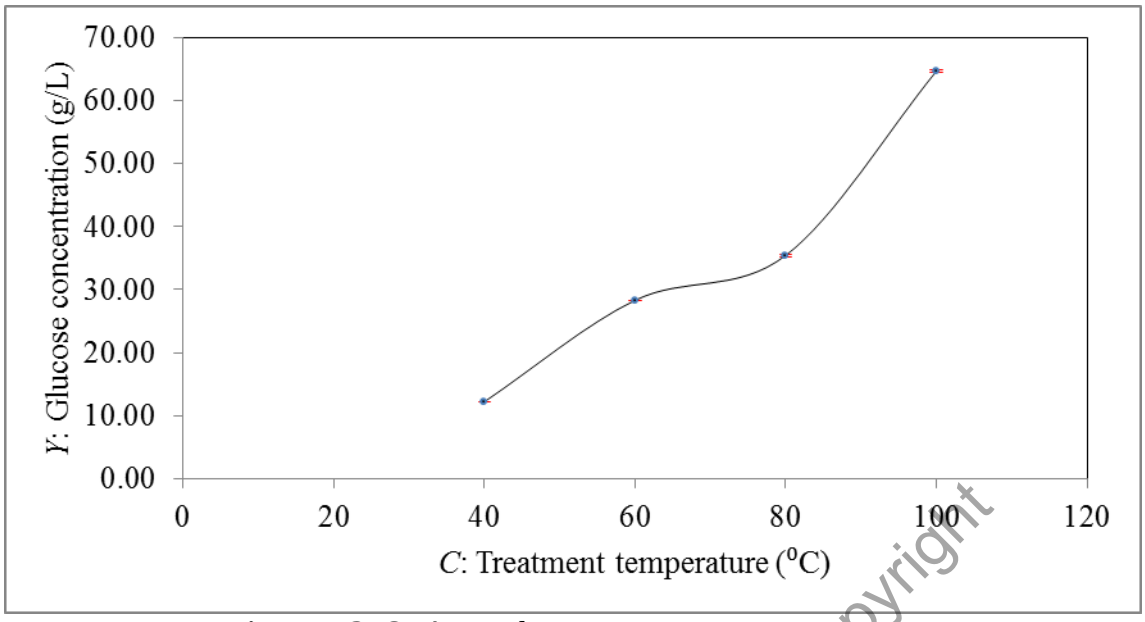

Figure 4.3: OFAT analysis on treatment temperature.

4.3 Optimization Studies: Response Surface Methodology (RSM)

Central Composite Rotatable Design (CCRD) was used to construct the response mode by inputting the experimental ranges and levels of independent process variables after preliminary optimum parameters studies in OFAT part. Treatment temperature was remain unchanged at 100 ℃ as fixed highest temperature to be apply in banana trunk biomass pretreatment studies in order to reduce the energy consumption at the same time eliminate the risk of enzyme inhibitors formation in acidic biomass pretreatment process. Table 4.3 shows the coded and actual values of the considered process parameters which are the substrate concentration and treatment duration.

Table 4.3: Coded and actual values of process parameters in CCRD.

Independent variable	Code	Unit	Level				
			-α	**-1**	**0**	**+1**	**+α**
Substrate concentration	*A*	% (w/v)	5.86	10.00	20.00	30.00	34.14
Treatment duration	*B*	minutes	22.93	25.00	30.00	35.00	37.07

Note: α = 1.1421 for CCRD (k < 6)

According to the OFAT studies, the minimum and maximum values chosen for substrate concentration were 10% (w/v) and 30% (w/v) respectively. On the other side, the minimum and maximum treatment durations were chosen to be 25 minutes and 35 minutes. Since the glucose responses curvature, it was a distinct possibility, an experimental design that allowed estimating a second order or quadratic model was needed. For only two considered factors, this design is typically recommended to have 13 runs with 5 center point runs.

Table 4.4 indicates the glucose response yields obtained using enzymatic hydrolysis with the combination of substrate concentration and treatment duration as the pretreatment process parameters. According to Table 4.4, it can be observed that experimental run number 11 (refer to red shaded row) with process condition of 20% (w/v) substrate pretreated in 37.07 minutes gave the highest glucose response value of 99.57 g/L in subsequent hydrolysis step; on the other hand, the experimental run number 8 (refer to blue shaded row) with process condition of 5.86% (w/v) pretreated in 30 minutes gave the lowest glucose response value of 31.30 g/L in subsequent hydrolysis step.

Table 4.4: Experiment complete design matrix with coded factors of CCRD and glucose response.

Run	Factor *A*: Substrate concentration (% (w/v))	Factor *B*: Treatment duration (minutes)	Glucose response value (g/L)
1	30.00	25.00	81.83
2	10.00	25.00	53.90
3	20.00	22.93	75.47
4	20.00	30.00	84.03
5	34.14	30.00	81.60
6	10.00	35.00	66.83
7	20.00	30.00	84.47
8	5.86	30.00	31.30
9	30.00	35.00	88.80
10	20.00	30.00	84.40
11	20.00	37.07	99.57
12	20.00	30.00	84.30
13	20.00	30.00	84.37

The finding of a great increase of substrate concentration (15.86% (w/v) differences) and small increase of treatment duration (7.07 minutes differences) causing a highly glucose production increment from 31.30 g/L to 99.57 g/L indicates that substrate concentration have a greater significant effect on the performance of biomass pretreatment. This can be explained by substrate which acts as the limiting reactant with chemical requires the optimum sufficient concentration and adequate mixing distribution to achieve high performance of biomass pretreatment process; or else, there would not to have any greatly effects undergoing any increment or decrement of treatment duration (Lopez-Arenas *et al*., 2010; Karimi *et al*., 2013).

4.3.1 Development of Regression Model

The result obtained from Table 4.4 was analyzed using analysis of variance (ANOVA). Table 4.5 shows the ANOVA analysis for response surface reduced quadratic model for glucose production from pretreated banana trunk biomass.

Table 4.5: ANOVA for response surface reduced quadratic model.

Source	Sum of squares	Degree of freedom	Mean square	*F* value	Prob > *F*
Model	3582.54	4	895.64	67.57	< 0.0001 [a]
A	1831.18	1	1831.18	138.14	< 0.0001 [a]
B	364.26	1	364.26	27.48	0.0008 [a]
AB	8.88	1	8.88	0.67	0.4368 [b]
A^2	1378.22	1	1378.22	103.97	< 0.0001 [a]

R^2 = 0.9731; Adjusted-R^2 = 0.9569; Predicted-R^2 = 0.8524; Standard deviation = 3.64; Mean = 76.99

[a] Significant at 95% confident interval. [b] Not significant at 95% confident interval.

By eliminating the insignificant parameter of B^2 – treatment duration (prob > *F* = 0.2069) in which the value of "prob > *F*" was greater than 0.05, multiple regression analysis provides the following quadratic model equations that correlates the glucose production to pretreatment parameters of substrate concentration (*A*) and treatment duration (*B*).

Quadratic model equation in terms of coded factors:

Glucose concentration (g/L) = + 85.58 + 15.13**A* + 6.75**B* – 1.49**A***B* – 13.96*A^2 (4.1)

Quadratic model equation in terms of actual factors:

Glucose concentration (g/L) = – 58.86869 + 7.98902*substrate concentration + 1.94556*treatment duration – 0.029800*substrate concentration*treatment duration – 0.13955*substrate concentration^2 (4.2)

According to Table 4.5, source *AB* with the prob > *F* value equal to 0.4368 which greater than 0.05 still retained and considered in the regression model analysis since source A^2 was significant with small prob > *F* value of < 0.0001 and the hierarchy principle supports that a higher order term (A^2) is the correction factor to the lower order term (*AB*) in which *AB* believed to have a significant interaction with A^2.

As shown in Table 4.5, the *F* test (Fisher) on Equation 4.1 and 4.2 gives an *F* value of 67.57 and a "prob > *F*" value of < 0.0001, indicating that the developed model is significant. Besides that, R^2 shown in Table 4.5 is very close to unity, 0.9731, indicating that the developed model equation successfully captured the correlation between process parameters to the production of glucose for pretreatment process of banana trunk biomass.

4.3.2 Analysis of Residual

The significant developed model equation was validated by performing a residuals analysis. Residuals refer to the differences between the actual glucose response value and the predicted response value; meanwhile, studentized residuals refer to the division of the residuals by the standard errors of the residuals for the adjustment accounts for different variances in the residuals.

Table 4.6 shows the diagnostics case statistics that listed the actual glucose response value, predicted glucose response value, residuals and internally studentized residuals. By referring to Table 4.6, all the residuals values are small (in the value range of ± 5) and unstructured; this indicates that the regression analysis has been successful in explaining the parameters of substrate concentration and treatment duration in pretreatment process have significant effect in subsequent glucose production in hydrolysis process according to the developed quadratic model.

Table 4.6: Diagnostics case statistics.

Standard order	Actual glucose response value (g/L)	Predicted glucose response value (g/L)	Residual	Internally studentized residual
1	53.90	48.26	5.64	2.445
2	81.83	81.49	0.34	0.145
3	66.83	64.73	2.10	0.909
4	88.80	92.01	-3.21	-1.390
5	31.30	36.27	-4.97	-2.153
6	81.60	79.06	2.54	1.099
7	75.47	76.03	-0.56	-0.197
8	99.57	95.12	4.45	1.553
9	84.37	85.58	-1.21	-0.356
10	84.40	85.58	-1.18	-0.347
11	84.03	85.58	-1.55	-0.456
12	84.47	85.58	-1.11	-0.326
13	84.30	85.58	-1.28	-0.376

Based on the normal plot of residual in Figure 4.4, the normality of the residuals was been promised since the residual values are plotted against a theoretical normal distribution in such a way that the points rely approximately on a straight line without any significant outliers or unusual features. Normally distributed residuals indicate that the acquired standard regression model was assumed to be having adequate usage in glucose response prediction based on the considered parameters in pretreatment process.

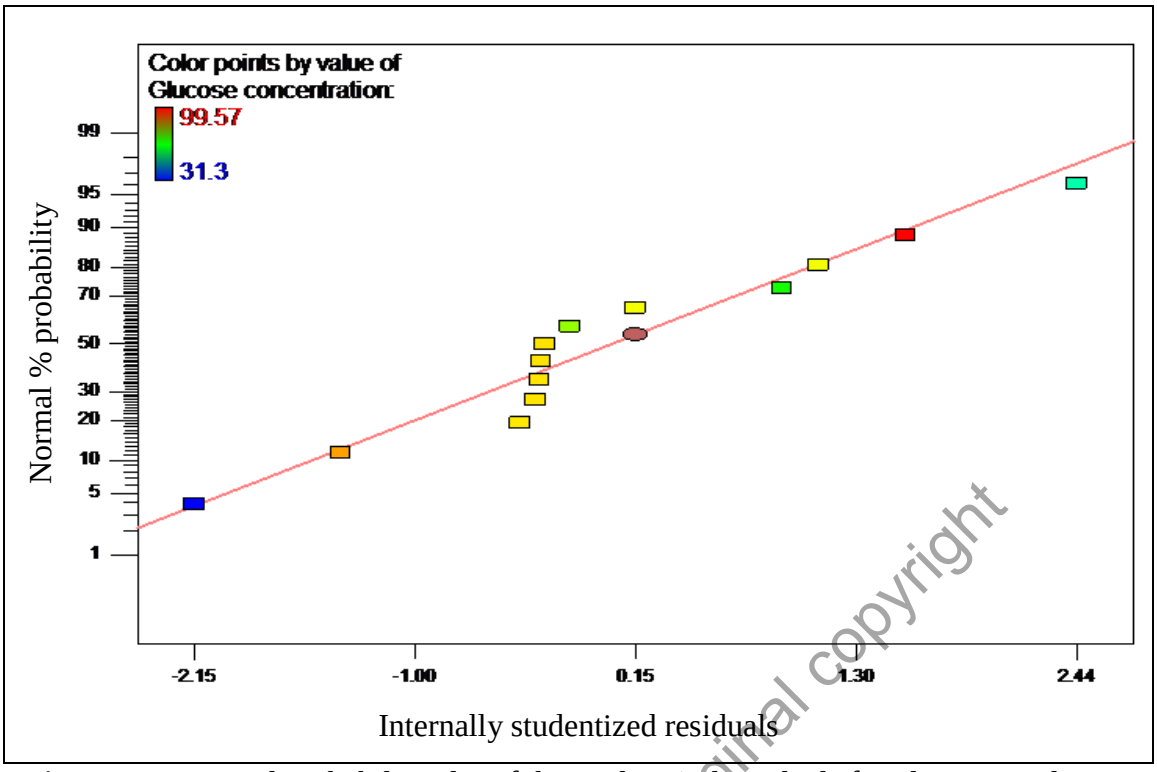

Figure 4.4: Normal probability plot of the studentized residuals for glucose production.

Suitability of the acquired regression model can be strongly confirmed again by checking the constant error through the graph of studentized residuals versus predicted response values as shown in Figure 4.5. A random scatter plot with almost constant range of residuals must be performed and Figure 4.5 proved it at the same time verified the significant of regression model since the residuals plot did not reveal any major violations of the underlying assumptions.

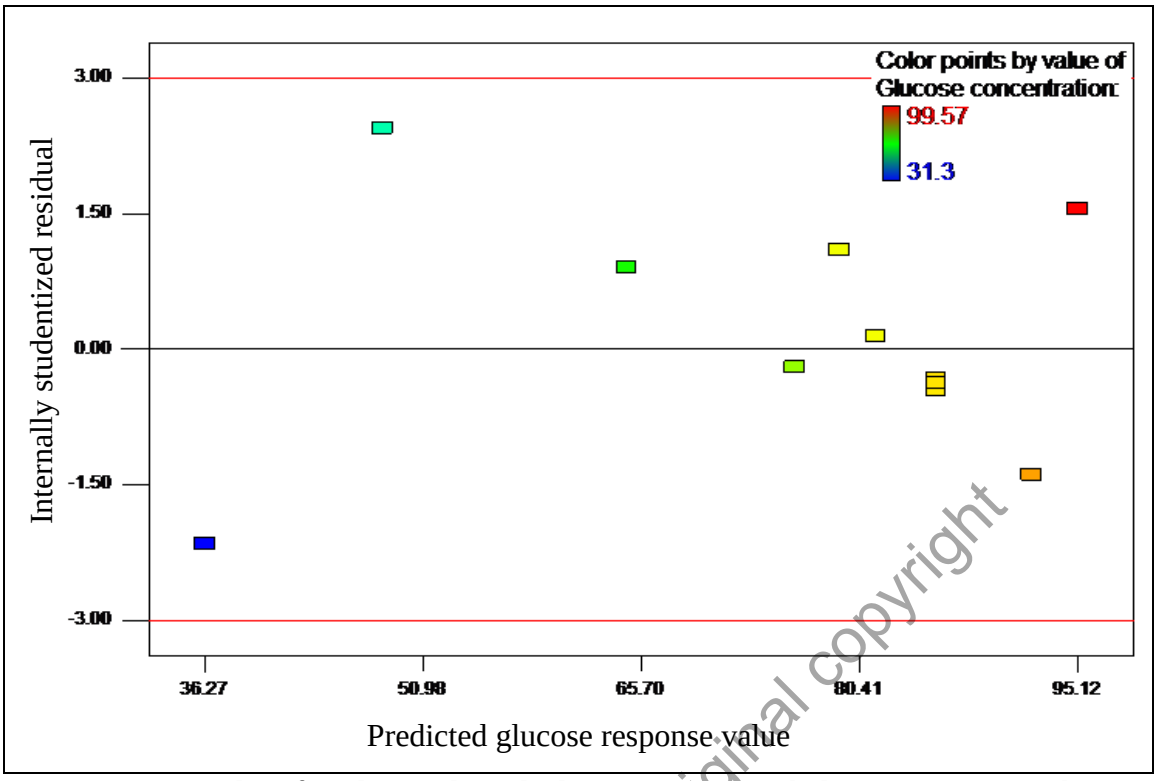

Figure 4.5: Plot of studentized residuals versus predicted glucose response value.

4.3.3 Analysis of Model

On the basis of the results shown in Table 4.5 (refer subtopic 4.3.1), the two process parameters studied, substrate concentration (*A*) and treatment duration (*B*) in acidic chemical pretreatment were found to significantly affect the glucose production in enzymatic hydrolysis because their *F* values (138.14 and 27.48 respectively) and "prob > *F*" values (< 0.0001 for both) were dropped at the 95% confidence interval. The parameter with the highest *F* value will have the most significant effect. Thus, by referring to *F* values mentioned before, substrate concentration was the parameter with the most significant effect on the glucose production compared to treatment duration.

Apart from that, the positive sign for both regression coefficients of *A* and *B* in equation 4.1 (refer subtopic 4.3.1) indicated a positive effect on the glucose production. These findings can be easily verified by visually inspecting the experimental results shown in Table 4.4 (refer subtopic 4.3). For example, by comparison between runs 1 and 2 as well as runs 6 and 9, an increase in substrate concentration in pretreatment

process caused a vast increase in glucose production in subsequent enzymatic hydrolysis. However, for treatment duration (runs 2 and 6 as well as runs 1 and 9), increases in this parameter value caused only a slight increment in the glucose production during enzymatic hydrolysis

Figure 4.6 shows the interaction effect between substrate concentration (*A*) and treatment duration (*B*) in pretreatment process on glucose production in enzymatic hydrolysis. The significant effects of both the factor *A* and *B* were evaluated and presented in three-dimensional response surface plot and two-dimensional contour plot as shown in Figure 4.6. From Figure 4.6, it can be seen that when substrate concentration is set at lower value range from 10% (w/v) to 15% (w/v), there is only a marginal increase in the glucose production in subsequent hydrolysis with longer treatment duration; however, when the substrate concentration is set at higher value range from 20% (w/v) to 25% (w/v), the glucose production in hydrolysis increases more significantly with longer treatment duration.

The finding and observation further strengthens the claim mentioned before that substrate concentration is a more prominent pretreatment process parameter that affects the glucose production in hydrolysis as compared to treatment duration. This can be justified that banana trunk biomass plays the most important role as reactant in the lignocellulosic structural modification process with the acidic (H_2SO_4) solvent (Lopez-Arenas *et al*., 2010). Therefore, by increasing the biomass as substrate in the reaction mixture, the more mass transfer of H_2SO_4 into the biomass matrix in enhancement of biomass structural disruption and porosity formation (Karimi *et al*., 2013).

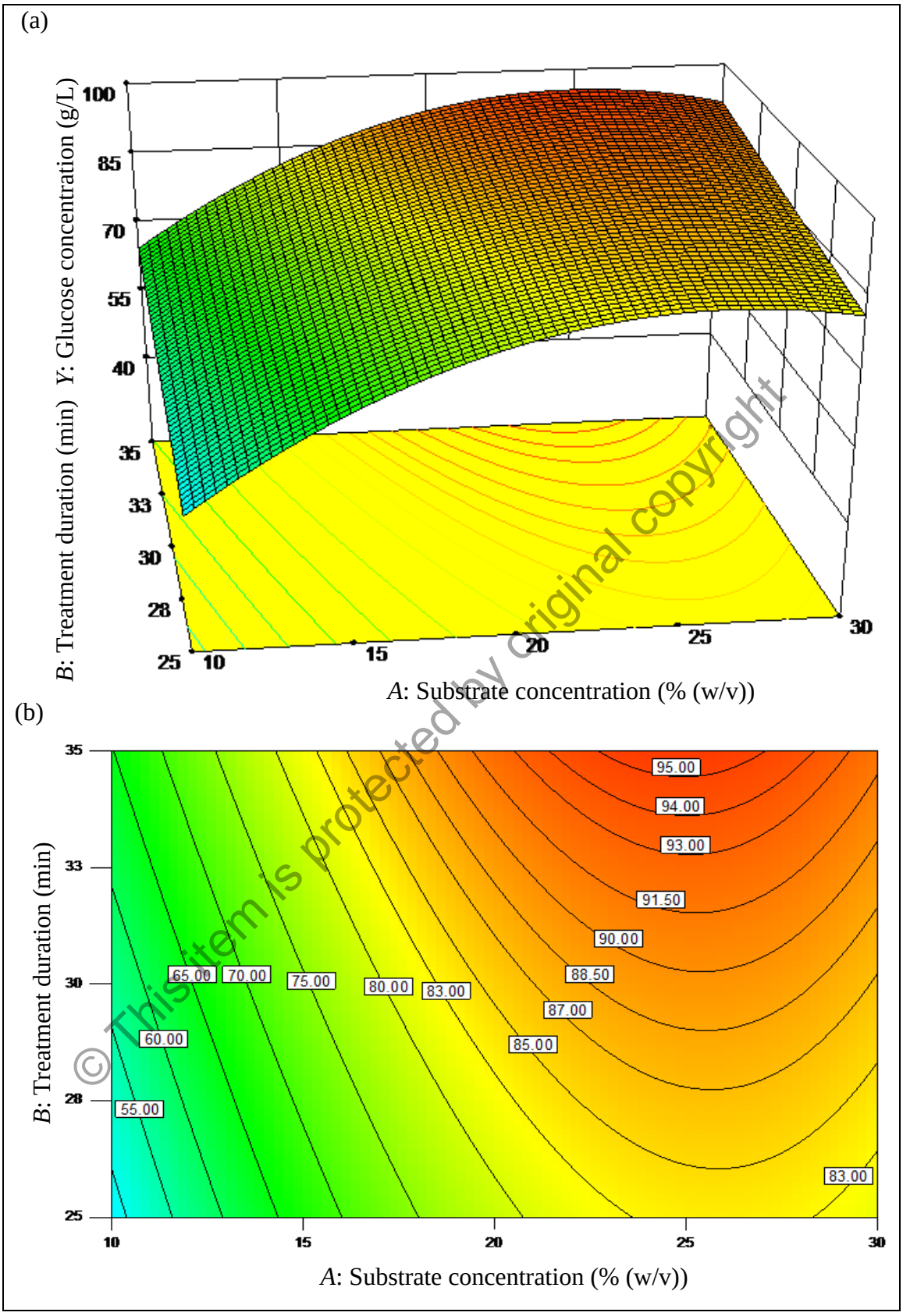

Figure 4.6: (a) Three-dimensional response surface plot of predicted glucose production and (b) Two-dimensional contour plot of predicted glucose production.

However, mass transfer limitation would occur when substrate concentration reach saturation in pretreatment process as it causes the decrease of liquid content due to high viscosity of reaction medium and further leads to non-homogeneous distribution between substrate and chemical solution for proper pretreatment reaction (Karimi *et al*., 2013). This phenomenon was started happening for substrate concentration onwards 25 % (w/v) as shown in Figure 4.6.

From Figure 4.6, it shows the glucose production was found to increase linearly with pretreatment duration in agreement with the previous studies made by Lopez-Arenas *et al*. (2010) and Lei *et al*. (2013). They reported that the limiting factor for glucose extraction in enzymatic hydrolysis after chemical pretreatment process is the biomass solubilization and reduction of biomass particle size (Myat and Ryu, 2015). Therefore, sufficient time will be required to depolymerize the lignocellulose and increase the porosity on the biomass and subsequently be hydrolyzed to glucose effectively.

4.4 Response Surface Optimization Design and Validation

Optimization of the process parameters has been carried out after the verification of the significant effects of substrate concentration and treatment duration in order to obtain the highest glucose production. Both of these individual process parameters including their interaction between parameters were taken into account in optimization procedure by using the developed reduced regression model as shown in equation 4.1 and 4.2 in subtopic 4.3.1. Table 4.7 shows the limits for each of the pretreatment process parameters used in the optimization procedure. The ramps set for all the pretreatment process parameters and glucose response also been shown in Figure 4.7.

Table 4.7: Constraints used to optimize the glucose production in hydrolysis step after pretreatment process.

Variable	Goal	Lower limit	Upper limit
A: Substrate concentration (% (w/v))	In range	10.00	30.00
B: Treatment duration (min)	In range	25.00	35.00
Y: Glucose concentration (g/L)	Maximize	31.30	99.57

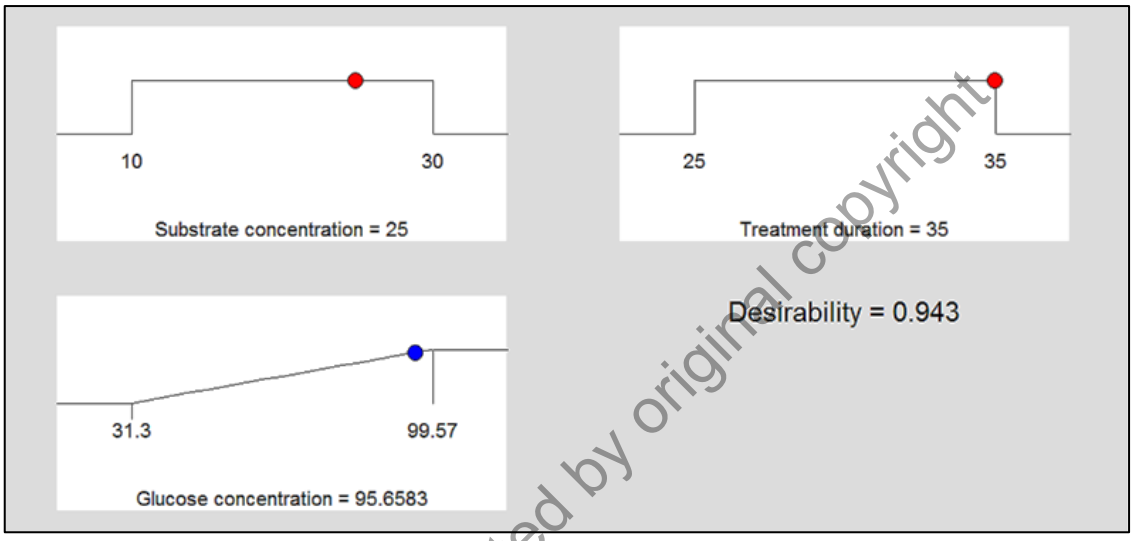

Figure 4.7: Ramps set for factors of substrate concentration and treatment duration together with glucose response in concentration.

According to the set goals for substrate concentration which in range from 10% (w/v) to 30% (w/v) and treatment duration which in range of from 25 minutes to 35 minutes while glucose response in response aimed to be maximized and optimized from the lower limit of 31.30 g/L to upper limit of 99.57 g/L, the Design-Expert software generated two optimal conditions solutions based on the 95% confidence interval as listed in Table 4.8.

Table 4.8: Optimal pretreatment conditions solutions provided by the Design-Expert software.

Number	Substrate concentration (% (w/v))	Treatment duration (min)	Glucose concentration (g/L)	Desirability
1	25	35	95.6583	1
2	26	35	95.5516	1

The yellow highlighted solution number 1 with desirability of 1 was selected since it was preferable to choose utilization of lower substrate concentration (25% (w/v)) to obtain higher glucose concentration (95.6583 g/L) at the same period of treatment duration (35 minutes) compared to the suggested solution number 2 (26% (w/v)). The predicted optimum glucose production (95.6583 g/L) was then verified by carrying out three repeated experimental runs using the suggested optimum condition. The repeated experiments gave an average optimum glucose production of 97.6900 g/L, which is very close to the predicted value (< 5% error – 2.07%), indicating that the predicted optimum pretreatment process conditions are valid for this research study.

It is noted here that although the optimum glucose production in hydrolysis was slightly lower (1.88%) than the production obtained in run 11 (refer Table 4.4 in subtopic 4.3), the optimum glucose production can be obtained from biomass that pretreated at milder reaction condition which requiring less pretreatment duration (6% reduction) in order to reduce the risk of enzymatic inhibitors formation in acidic solution (H_2SO_4). It was found that the optimum substrate concentration (25% (w/v)) in dilute acidic (2% (v/v) H_2SO_4) pretreatment of banana trunk biomass to obtain optimum glucose production in subsequent enzymatic hydrolysis in this study was proved the general statement made by Harmsen *et al*. (2010) which stated that most of the lignocellulosic materials are suggested to utilize substrate concentration range from 10% (w/v) to 40% (w/v) at temperature lower than 160 ℃ (100 ℃ was applied in this study) for weak acid pretreatment (in the range of 2% (v/v) to 5% (v/v)) in order to prevent the toxic compound formation which referred as enzymatic inhibitors that significantly influence the performance of enzymatic hydrolysis for optimum glucose production.