Reducing Sugar Production in Subcritical Water and Enzymatic Hydrolysis using Plackett-Burman Design and Response Surface Methodology

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***Abstract*— Subcritical water is one method of hydrolysis that can convert coconut husk to produce reducing sugars. However, this method has the disadvantage of producing derivative products such as furfural and phenolic compounds that act as inhibitors. One effective method is the addition of additives to the subcritical water process. The purpose of this study was to determine the effect of adding additives to subcritical water processes and optimizing the operating conditions on the production of reducing sugars. The analysis of reducing sugar was conducted by the dinitrosalicylic acid (DNS) method. Variables used in this study were time, temperature, pressure, water volume, pH, and several types of additives. Plackett-Burman was used for screening significant factors for the production of reducing sugars. The three most affecting factors were further investigated to find out the optimum point using Response Surface Methodology (RSM). The optimum point for subcritical water pretreatment operating conditions was the addition of Sodium Dodecyl Sulfate (SDS) of 0.24 grams, reaction time for 80 minutes, and pH 11 yielding a reducing sugar yield of 22.7%, energy use of 291.3 kJ/g with desirability of 85%. Furfural content of all liquids after pretreatment can be neglected (<2 ppm) because of the effect of surfactant.**

***Keywords*— coconut husk, plackett-burman, response surface methodology, reducing sugar, and subcritical water.**

I. INTRODUCTION

SUBCRITICAL water (SCW) hydrolysis considered as greener alternative for the hydrolysis of lignocellulosic biomass. SCW can be used to dissociate cellulose and hemicelluloses from the linkages of the lignocellulose. SCW also reduce the lignin content of the biomass by breaking ether and ester bonds of lignin and hemicellulose [1] Compared to another type of hydrolysis, SCW need shorter time interval, does not require solvent separation, and has minimal corrosion. [2] However, SCW hydrolysis also has disadvantage, it requires high energy in the process that can increase the cost. Therefore, the optimization of the process is needed with enhancing efficiency of the conversion.

Several techniques including varying operating conditions and adding additives has been done to aim the maximum yield of produced sugar. SCW hydrolysis with varying operating condition of pressure, water volume, severity factor [3] [4] [5] and the addition of surfactant [6] were obtained significant results. However, the effe ct of operating condition and the addition of surfactants simultaneously has never been studied and discussed. The studies that have been conducted also using OFAT (One Factor at A Time) that is time-consuming method and the interaction between factors cannot be determined. Some studies involved the use of design experiment has been reported to save the time and minimizing error in the experiments [7]. Therefore, the use of design experiment in the study of SCW hydrolysis has never done and it interestingly enough to study.

From the problems mentioned above, the present work aims to maximize the sugar yield with low furfural content and reduced energy use and also study the effect of SCW hydrolysis to enzymatic hydrolysis. The significant factors, optimum operational condition and the effect of SCW hydrolysis to enzymatic hydrolysis were investigated using statistical approach. The SCW hydrolysis were investigated using statistical approach with Plackett-Burman Design (PBD) and Response Surface Methodology (RSM), the yield, energy and furfural content were assessed. The long-term goal of this study is with the optimum condition of SCW hydrolysis, it could make the hydrolysis more feasible and can be done on industrial scale.

II. METHOD

A. Materials

CCH (120 mesh) was obtained from Manado, North Sulawesi, Indonesia. Commercial surfactants (SDS, CTAB, and Tween 80) as additives in SCW were purchased from Merck, Germany. While commercial cellulase enzyme from Aspergillus niger and xylanase enzyme from Trichoderma longibrachiatum were purchased from Sigma Aldrich, Japan.

B. Subcritical Water Pretreatment

SCW pretreatment use the same apparatus as the previous work [1]. The amount of CCH in the pretreatment process is 6 grams. Deionized water, surfactant, and pH (by adding NaOH) are adjusted according to the level of experimental design used and then mixed with CCH in the reactor. To get the desired pressure, ultra-high purity carbon dioxide (CO_2) (PT. Aneka gas, Sidoarjo, Indonesia) was supplied to the reactor. Temperature and time were also adjusted in this pretreatment. After that, reactor was cooled to ambient temperature. The pretreated solid samples were separated from the liquid and dried in an oven at 60 °C for 2 d without washing and neutralization. Solid and liquid samples are stored at 4 °C before analysis.

C. Plackett-Burman Design (PBD)

PBD which consists of 12 experimental designs is based on the first order model. This experimental design is an efficient way to streamline the screening of factors that are significant to produce reducing sugar among a large number of factors. The factors to be screened at SCW pretreatment consisted of SDS, CTAB, Tween 80, pH, temperature, time, pressure, and volume of deionized water. Based on the experimental design of Plackett-Burman, two levels (-1 for low level and +1 for high level) are determined for each factor (Table 1). The reducing sugar yield is calculated by the following equation [18] :

$$\%TRS = \frac{sugars, g}{(cellulose + hemicellulose), g} \quad (1)$$

Design matrix of the level and response of the experimental designs that have been investigated is presented in Table 2. After finding the 3 most significant factors in sugar production, the experiment continued with optimization using central composite design.

Table 1.
Levels of The Variables and Statistical Analysis of Plackett–Burman Design

Code	Factors	Low level (-)	High level (+)	Effect	P value
X_1	SDS (g)	0.06	0.18	1.872	0.156
X_2	CTAB (g)	0.06	0.18	-2.785	0.068
X_3	Tween 80 (g)	0.06	0.18	1.713	0.183
X_4	Temperature (°C)	120	150	1.029	0.376
X_5	Pressure (bar)	20	60	0.521	0.636
X_6	Time (h)	20	60	1.467	0.236
X_7	Water Volume (ml)	100	140	1.268	0.291
X_8	pH	6	8	2.002	0.137

Table 2.
The Plackett–Burman Experiment Design Matrix and Experimental Results

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	%TRS
1	-1	1	1	1	-1	1	1	-1	13.49
2	1	-1	-1	-1	1	1	1	-1	21.38
3	1	-1	1	-1	-1	-1	1	1	21.67
4	1	-1	1	1	-1	1	-1	-1	24.10
5	-1	-1	1	1	1	-1	1	1	28.41
6	-1	-1	-1	1	1	1	-1	1	16.70
7	-1	-1	-1	-1	-1	-1	-1	-1	9.43
8	1	1	-1	1	-1	-1	-1	1	13.24
9	1	1	-1	1	1	-1	1	-1	11.50
10	-1	1	-1	-1	-1	1	1	1	12.92
11	-1	1	1	-1	1	-1	-1	-1	2.92
12	1	1	1	-1	1	1	-1	1	22.40

D. Central Composite Design (CCD)

Significant factors generated from Plackett-Burman were then optimized using CCD with six replicates at the center point. The operating conditions in CCD for each factor were conducted at three levels (−1; 0; +1;) plus two α levels (-1.68; +1.68). In the optimization process, the desired optimum point in the liquid sample after SCW pretreatment consists of high sugar yield. Low energy and low furfural content also considered. Quadratic equation is used as a fitting model to represent relationships between factors.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response, β0 is the constant coefficient, βi is the linear coefficients, βii is the quadratic coefficients, βij is the interaction coefficients and Xi, Xj (i=1,3; j=1,3; i≠j) were the independent factors. Minitab 16 statistical software (Minitab Inc., ITS Surabaya, Indonesia) was used to analyze experimental designs and create three dimensional surface plots. Then the results of ANOVA (Analysis of Variance) are optimized with the highest level of importance is the sugar yield while energy and furfural are equal. The factors and their levels were presented in Table 3.

E. Enzymatic Saccharification

A total of 1 g of solid sample produced from SCW pretreatment (in the CCD experimental design) was hydrolyzed using cellulase combined with xylanase of 18.6 U/g respectively. Then citrate buffer 0.1 M pH 3 was added to the enzyme solution and a solid sample of up to 30 mL. The solution was incubated at a temperature of 60 °C and 125 rpm. The sugar concentration was analyzed after 2 hours, 4 hours, 8 hours, 16 hours, 32 hours, and 48 hours.

F. Analysis

The concentration of reducing sugars produced from SCW and enzymatic saccharification was measured based on the dinitrosalicylic acid (DNS) method using Vispectrophotometer (CECIL 1001, Cambridge, UK). Furthermore, furfural content in liquid samples was analyzed using gas chromatography-flame ionization detection (GC-FID) (GC-2010 Plus Shimadzu, Kyoto, Japan) [8]. Furfural measurements have been modified which refer to previous work [1]. The specific energy (kJ/CCH) as one of the SCW responses was monitored using the PZEM 061 kWh meter (Peace Fair, China) installed at the SCW reactor. pH (as a factor) before and final pH (in the liquid sample) after pretreatment was measured using a portable pH meter (Starter 300, Ohaus, Canada).

III. RESULTS AND DISCUSSION

A. Experimental Design and Statistical Analysis

1) The Most Influential Screening Factor

Plackett-Burman Design is a two-level factorial screening design for studying 4n-1 variables using 4n runs so to investigates 8 factors, it needs 12 runs. The positive effect of the responses means that the reducing sugar yield are higher on the high level (+1), while the negative effect means the reducing sugar are higher on the low level (-1). Responses produced by PBD experiments that has been done shown in Table 1. Experiment result shows that factor X_1 (SDS), X_3 (tween 80), X_4 (temperature), X_5 (pressure), X_6 (time), X_7 (water volume), and X_8 (pH) give positive effects to reducing sugar yield. Whereas X_2 (CTAB) gives negative effect to reducing sugar yield. The decreasing of reducing sugar could happen because the more concentration of CTAB, the polarity effect would be greater than electrostatic effect, CTAB made the condition more nonpolar so the reaction rate of substrate degradation would be decreased [9]. [10] also proved that CTAB has the effect to decrease the yield of enzymatic hydrolysis in larger concentration.

Generally, PBD screening process with the negative effect factors are handed down little by little in low level area, factors with positive effect are raised little by little in the high level area using steepest ascent, and keep the insignificant factors on the level that produce biggest effect [11]. However, in this work only one type of surfactant was chosen

based on highest positive effect, while two other surfactants are not used on the optimization process using CCD. Based on p-value, CTAB was the most significant surfactant with the p-value of 0.068. However, CTAB has the negative effect for the reducing sugar production on SCW process. After CTAB, SDS was the most significant factor with p-value of 0.156 with positive effect. Therefore, only SDS is selected as additive that used on the optimization process using CCD. From three types of surfactants, it can be concluded that CTAB is the cationic surfactant that has inhibition effect while Tween 80 which is nonionic surfactant not significant as much as SDS which is the anionic surfactant in increasing reducing sugar production on the SCW process. Besides SDS, factors selected which having smallest p-value are F (time) and H (pH) that is equal to 0.236 and 0.137 respectively for the next optimization using CCD.

2) Optimization of The Significant Factors

The untreated coconut husk consists of 20.05% of hemicellulose, 16.90% of cellulose and 51.30% of lignin [12]. Because of the high content of lignin, it needs more effort to get a high conversion of reducing sugar. From the screening process using PB, the significant factors obtained made the pretreatment process a combination of SCW-pretreament and alkali- pretreatment (pH factor) in the one pot process of SCW. On the SCW process, most studied responses are reducing sugar yield (%TRS) and side products like furfural from the extension degradation of reducing sugar. [1] added energy study from SCW process with the addition of surfactants to make this process more efficient. Therefore, SCW hydrolysis optimization process from this work investigates three significant variables which are SDS, time, and pH towards multiple responses which are yield and energy using CCD. From the two responses, yield response was prioritized over the energy responses on the optimization process. Design and experimental result responses were shown on the Table 3.

Multiple regression analysis fitted on the experimental data and the predicted response of %TRS could be obtained by the quadratic polynomial equations below:

$$\%TRS = 45.9 - 162.5X_1 - 0.082X_6 - 4.20X_8 + 93X_1^2 - 0.00173X_6^2 + 0.235X_8^2 + 1.326X_1X_6 + 4.55X_6X_8 + 0.0095X_6X_8 \quad (3)$$

Where X_1, X_6, and X_8 are, in terms of coded factors of SDS, time, and pH, respectively.

The competence of developed model was analyzed by ANOVA and the results were shown in Table 4. A p-value of lack of fit were 5.5097 for yield proposing that the model was insignificantly relative to pure error [13]. For yield response, time and pH were significant compared to SDS. Time and pH has the most significant effect to reducing sugar yield because the two factors have a role in the degradation reaction, whereas SDS only act as lignin bonding agent so polymerization won't happen again [3].

Based on the developed model by RSM, the 3D surface plots and contour plots of the model were generated to show the effects of factors and their interaction. A total of three set of plots were generated and each plot showed the effects and interaction of two most significant factors while the third factor was set in zero level

Table 3.
Original and Coded Values of The Independent Factors and CCD Matrix Along with The Experimental Responses

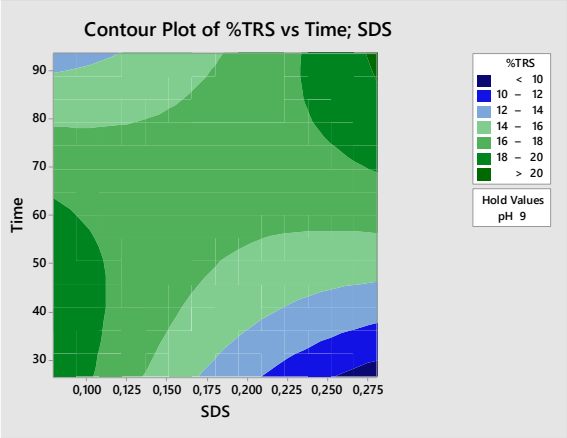
Runs	Factors (Coded)			Responses			
	X_1	X_6	X_8	TRS (%)	TRS Predicted (%)	Energy (kJ/g)	Energy Predicted (kJ/g)
1	0.12	80	7	15.01	14.14	241	252
2	0.18	60	9	15.87	16.57	288	261
3	0.18	60	9	19.39	16.57	252	261
4	0.18	60	9	16.63	16.57	259	261
5	0.18	93.636	9	15.39	15.81	296	288
6	0.18	60	5,636	14.16	14.49	253	246
7	0.24	80	11	25.47	22.69	286	291
8	0.12	40	11	20.91	20.44	221	226
9	0.079	60	9	20.72	18.38	252	244
10	0.18	60	9	16.34	16.57	257	261
11	0.12	40	7	14.06	16.66	224	224
12	0.24	80	7	14.92	15.2	281	282
13	0.18	26.364	9	13.59	13.42	203	203
14	0.18	60	9	15.01	16.57	256	261
15	0.24	40	7	14.06	11,36	223	228
16	0.18	60	12,364	24.04	23,97	256	255
17	0.28	60	9	14.06	16,66	263	263
18	0.24	40	11	16.63	17,33	223	218
19	0.12	80	11	16.91	19,44	272	273
20	0.18	60	9	16.25	16.57	252	261

Table 4.
ANOVA for the adjusted model of response

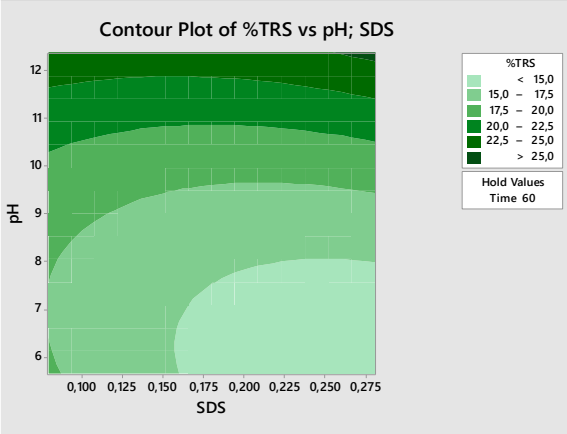
Source	SS	DF	MS	F Value	P Value
Model	23.8673	9	2.6519	3.82	0.024
X_1	0.3113	1	0.3113	0.45	0.518
X_6	0.6826	1	0.6826	0.98	0.345
X_8	15.9172	1	15.9172	22.92	0.001
X^2_1	0.1426	1	0.1426	0.21	0.660
X^2_6	1.1231	1	1.1231	1.62	0.232
X^2_8	1.5028	1	1.5028	2.16	0.172
$X_1 X_6$	3.4213	1	3.4213	4.93	0.051
$X_1 X_8$	0.1481	1	0.1481	0.21	0.654
$X_6 X_8$	0.3404	1	0.3404	0.49	0.500
Error	6.9439	10	0.6944		
Lack of Fit	5.5097	5	1.1019	3.84	0.083
Pure Error	1.4342	5	0.2868		
Total	30.8112	19			

. Every independent variables in this work was limited to its maximum value in the coded value of +1. SDS limited to 0.18 g w/w CCH because of the bubble produced will increase the pressure of the process significantly. In order to avoid higher degradation of %TRS, time was limited to 80 minutes and to keep the reactor safe from the corrosion, pH was limited to 11. Two factors from overall effects were plotted in contour plot as the independent variables and the third factor was hold at its zero level with %TRS as the main response (Figure 1). Energy and predicted values were shown in Table 3. The highest %TRS response was 25.47% obtained from coded value (+1) of SDS, time and pH. The significant independent variables of %TRS was pH and time but the significant interaction was SDS with time (Table 4). Those condition were confirmed because many scholars reported that the NaOH concentration (pH factor) have a strong effect on depolymerization of lignocellulose biomass. NaOH has a strong effect on lignin removal compared to time and temperature [17]. The greater the level of delignification will help the enzymatic process is easy to independent variables of %TRS was pH and time but the significant interaction was SDS with time (Table 4). Those condition were confirmed because many scholars reported that the NaOH concentration (pH factor) have a strong effect on depolymerization of

lignocellulose biomass. NaOH has a strong effect on lignin removal compared to time and temperatur [14] [15]. The greater the level of delignification will help the enzymatic process is easy to reach cellulose crystals and increase the yield produced. Figure 1b show that the significant factor with the time of 60 minutes was pH. The %TRS response above 25% will obtained from pH above 11. However, this condition is not allowed because of the corrosion effect. So, the optimum %TRS response that can be obtained from SCW-pretreatment was 22.7% with the independent variables were 0.24 g of SDS, 80 minutes of time and 11 of pH.



(a)



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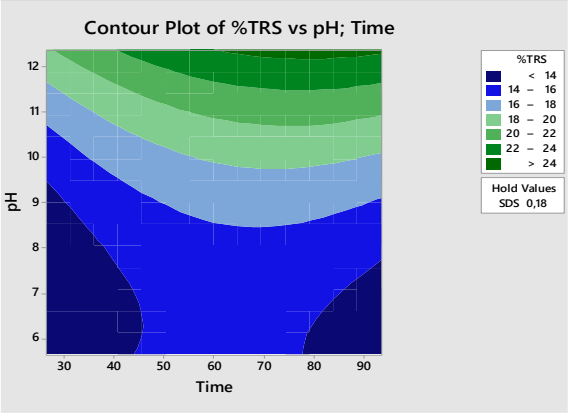

(c)

Figure 1. Response surface plots representing combined effects of variables on %TRS

SDS interaction with time tends to give %TRS which is high in the diagonal area with the highest value (%TRS above 20% w/w) at the SDS concentration below 0.1 g; time under 60 minutes and SDS above 0.23 g; time above 70 minutes (Figure 1a). This shows that the proportion of SDS and time is proportional to getting a high %TRS. While at Figure 1b and 1c showed that at high pH levels, SDS and time did not have a significant effect on changes in %TRS. In this study, to produce highest yields need the residence time between 60-80 minutes because of the lignin content in coconut fiber was up to 51.30%. Long residence times increase the formation of total reducing sugars but need to be monitored to minimize further degradation [16].

The main effect of each variable is shown in Figure 2. SDS has a tendency to reduce the yield of reducing sugars at a concentration of 0.22 g w/w CCH. In the process of adding SDS and pH, the excess SDS will maintain pH until the mole of NaOH added to regulate pH is greater than the SDS mole. Under these conditions, it can be assumed that only a small amount of Na+ is released from SDS so that the SDS surfactant in the form of negative ions decreases.

Figure 2. Main effect of each factors of CCD

3) Specific Energy Required in Subcritical Water

For the purpose of minimizing production costs, specific energy on SCW needs to be monitored. Multiple regression analysis fitted on the experimental data and the predicted response of energy required on SCW process could be obtained by the quadratic polynomial equations below:

$$Energy = 96 + 235X_1 + 0.8X_6 + 14.9X_8 - 724X_1^2 - 0.0139X_6^2 - 0{,}917X_8^2 + 5.52X_1X_6 - 24.0X_1X_8 + 0.122X_6X_8 \quad (4)$$

Where X_1, X_6, and X_8 are, in terms of coded factors of SDS, time, and pH, respectively. For energy response, time was the most significant factor compared to two other. This is because SDS and pH have low specific energy so that the increase in SDS concentration is not so significant in increasing energy requirements [1]. From table 4 it can be seen that time is a very significant factor in increasing energy needs (p-value<0.05), while SDS and pH have p-values of 0.115 and 0.393, respectively.

4) Furfural Presence in Subcritical Water

Furfural is formed due to degradation of xylose. This degradation occurs when the xylose ring is open due to the acyclic mechanism or two directly different cyclic mechanism [17]. On several literature, longer reaction time would make the degradation of reducing sugar becoming furfural component more likely. This happens because the longer the time, the severity of the degradation of the polymer that continue to degrade the monomers into products such as furfural and 5-HMF also higher [18]. The presence of furfural and 5-HMF can be an inhibitor of the enzymatic process [19]. But so far the effect has not been observed on pretreatment processes such as subcritical water. While 5-HMF is a

compound that is soluble in water so it cannot be captured by the hydrophobic side of surfactants. Because of the effects of the continued degradation, the longer the pretreatment will cause a detrimental effect on the final yield. In [19], furfural produced reached 36.32 ppm at a temperature of 180 °C with 4% sulfuric acid assisted microwave pretreatment so that its concentration needed to be reduced so as not to interfere with enzymatic hydrolysis. However, furfural detected in this study was not too much (<2 ppm) where the amount did not significantly affect enzymatic hydrolysis so that it could be ignored (Figure 3). This condition is possible because furfural has been trapped into surfactant micelles due to the solubility of furfural in water.

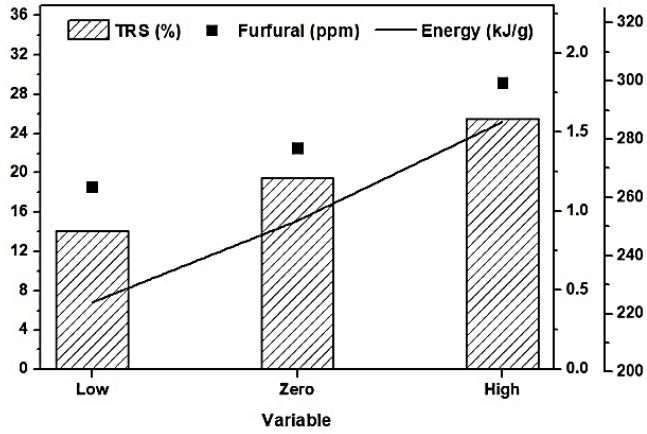

Figure 3. Comparison of %TRS, energy and furfural at level -1, 0, and +1

B. Enzymatic Saccharification and Total Reducing Sugar

Solids formed from the results of subcritical water pretreatment experiments are further hydrolyzed to maximize the production of reducing sugars and find out the effect of their pretreatment on enzymatic hydrolysis.

At long incubation times, the yield of reducing sugars tends to be constant or low. This tendency is made possible by the inhibitor of the end product that accumulates, causing a decrease in enzyme activity. In this study, reducing sugar in the largest enzymatic hydrolysis (36.01%) occurs in the SDS factor of 0.24, time of 40 minutes, and pH of 7. This indicates that SDS is the most influential factor in increasing the production of reducing sugars in the enzymatic hydrolysis process. SDS is a surfactant that serves to reduce the exposure of inhibitors to the substrate and dissolve the substrate into the buffer so that the enzyme can more easily reach the substrate [1] While pH does not have a significant contribution to the enzymatic process of hydrolysis. The results of research conducted by [1] also confirmed that enzyme activity decreased continuously during incubation at 60 °C within 6-8 hours. Although the highest enzymatic hydrolysis results were at high SDS concentrations, the largest total %TRS (58.44%) was located at SDS of 0.18, time was 60 minutes, and pH was 12.36 where %TRS of SCW and enzymatic was 24.04% and 34.40% respectively. This proves that pH is very influential on the SCW process while SDS is very influential on the enzymatic hydrolysis process

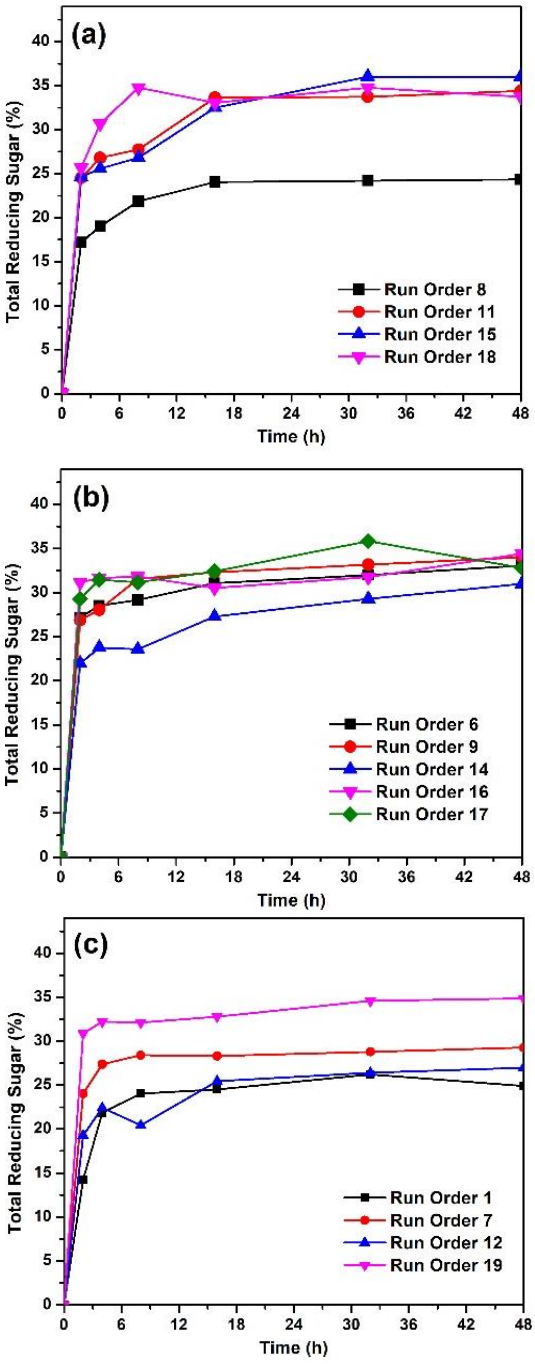

Figure 4. Enzymatic saccharification of each run order

IV. CONCLUSION

Response surface methodology is used to analyse optimum point of significant variables that obtained from Plackett-Burman method efficiently with polynomial model. Based on Plackett-Burman analysis, SDS, time and pH variable were the most significant factors and %TRS optimum that obtained from CCD was 22.7%, energy use of 291.3 kJ/g with desirability of 85% on the Sodium Dodecyl Sulfate (SDS) of 0.24 grams, reaction time of 80 minutes, and pH 11. However, reducing sugar in the largest enzymatic hydrolysis (36.01%) occurs in the SDS of 0.24 grams, time of 40 minutes, and pH of 7. This proves that pH is very influential on the SCW process while SDS is very influential on the enzymatic hydrolysis process.

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