Design and Performance of Amphiphilic Lignin Derivatives in Enzymatic Hydrolysis of Sweet Sorghum Bagasse for Bioethanol Production

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Sweet sorghum bagasse (SSB) is potential feedstock for bioethanol production due to its natural abundance and high cellulose content (> 40%). This work compared the impact of three variables relative to the enzymatic hydrolysis of SSB kraft pulp. The three variables were the biosurfactant from lignin derivative known as amphiphilic lignin derivatives (A-LD), the enzyme loading level, and the hydrolysis time. These variables were optimized by response surface methodology (RSM) with a Box-Behnken design (BBD). The concentration of polyethylene glycol (PEG) 4000 was also optimized to compare it with the A-LD performance in the enzymatic hydrolysis process. After optimization, the A-LD produced a higher reducing sugar yield (RSY) (99.45%) than the PEG 4000. The difference in the predicted *versus* experimental values of the RSY was less than 4%, which means that the model was highly predictive. The adequacy of the model was confirmed by a regression value close to 1 for the A-LD assisted test. The result implies that the A-LD significantly improved the enzymatic hydrolysis performance to enhance the RSY. Moreover, the BBD is adequate and useful to identify the optimum concentration of surfactant.

Keywords: Amphiphilic lignin derivatives (A-LD); Box-Behnken design; Enzymatic hydrolysis; Sweet sorghum bagasse (SSB)

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INTRODUCTION

Sorghum bicolor L. Moench, known as sweet sorghum bagasse (SSB), is abundantly present in Indonesia (Arif *et al*. 2019). The cellulose content of SSB is greater than 40%, which makes SSB a promising material to convert as bioethanol (Fatriasari *et al*. 2015). Therefore, many scientists have conducted research on the pretreatment of SSB using chemical (Jiang *et al*. 2019), physical, (Pramasari *et al*. 2017) and enzymatic applications (Yesuf and Liang 2014; Partida-Sedas *et al*. 2016). To enhance the reducing sugar, additives such as 2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol (Triton-X) (Eriksson *et al*. 2002), polyoxyethylene sorbitan monooleate (Tween) (Wang *et al*. 2018; Nababan *et al*. 2020), bovine serum albumin, Brij-35 (Rocha-Martín *et al*. 2017), and lignin derivative products resulting from the reaction of lignin with polyethylene glycol (Lin *et*

al. 2015; Chen *et al*. 2016; Fatriasari *et al*. 2020a,b) were added during the enzymatic process. This is because the hydrophobic interaction of the surfactant with lignin improves the non-productive binding of enzyme with cellulose, as well as the non-specific hydrophobic interaction with the lignin in biomass as the substrate (Uraki *et al*. 2001). There was also a competition of hydrophobic interaction between protein and surfactant, which can reduce the possibility of unfruitful enzyme bonding. The mechanism of resolution involves the ability for the surfactant to prevent the occurrence of a reconnection between the enzyme and the lignin, subsequently increasing the chance of enzyme reusability by immobilization (Börjesson *et al*. 2007).

Lignin is one of major compounds in lignocellulosic material, after cellulose. In Indonesia, approximately 0.41 million tons per year of lignin is produced as a byproduct of unutilized black liquor in the pulp and paper industry (Fatriasari *et al*. 2020a). The presence of lignin can interfere with enzyme adsorption, so the optimal release of sugars content cannot be achieved during the hydrolysis process. However, lignin itself can be converted to valuable products, such as non-ionic surfactants (Agrawal *et al*. 2014). This conversion can also enhance the overall efficiency process in terms of biorefinery concepts such as in the pulp and paper industry and for bioethanol production. Hermiati *et al*. (2017) isolated lignin with one and two step isolation methods from *Acacia mangium*. The black liquor produced a maximum lignin yield of 45% and an acid-insoluble lignin (AIL) percentage of 53%. Fatriasari *et al.* (2018) reported the conversion of lignin to the non-ionic surfactant, amphiphilic lignin derivative (A-LD). The addition of A-LD in enzymatic hydrolysis has been found to successfully increase the reducing sugar yield (RSY) of SSB kraft pulp up to 81%. Another study on the optimization of A-LD synthesis conditions also confirmed the beneficial effects of the RSY (Fatriasari *et al*. 2020a). The lignin derivatives addition was effective to increase RSY on several pretreated biomass containing low lignin content (Fatriasari *et al*. 2020b). Lin *et al*. (2015) compared enzymatic hydrolysis of corn stover with assisted lignin-based polyoxyethylene ether (EHL-PEG) and PEG. It was found that EHL-PEG was more effective than the PEG in improving the enzymatic hydrolysis. The glucose yield increased from 16% to 70% with the addition of EHL-PEG due to minimizing the nonspecific adsorption of the enzyme on lignin.

One obstacle in the bioethanol conversion process is the uneconomical hydrolysis enzymatic step because of the non-optimal conditions. For instance, the accessibility of enzymes was hampered in the presence of lignin, inappropriate enzyme loading, and non-optimal hydrolysis time. Surfactant plays role on reduction of enzyme adsorption onto lignin which will improve enzymatic hydrolysis. Therefore, determining optimal values such as the surfactant concentration, the enzyme loading, and the hydrolysis time have been determined to be influencing factors for the enhancement of the RSY (Eckard *et al*. 2011; Meyabadi and Dadashian 2012; Singh and Bishnoi 2012).

In this study, A-LD was synthesized using a set of optimized conditions and steps that were established in the authors' previous study (Fatriasari *et al.* 2020a). Subsequently, response surface methodology (RSM) with Box-Behnken design (BBD) was used to identify the contribution of the three-level variables on the RSY as a response. To date, there have been no published studies on the optimization of A-LD addition on enzymatic hydrolysis of pretreated biomass especially SSB by RSM with BBD as experimental design. Currently, BBD has been used by researchers to optimize the hydrolysis reaction time, the amount of enzyme, the sample loading, the hydrolysis temperature, and the surfactant concentration (Das *et al*. 2015; Delfín-Ruíz *et al*. 2019; Guarneros-Flores *et al*. 2019). The BBD is known to be more efficient than other response surface designs because

it only contains low, central, and high factor combinations. In other words, this design avoids performing the experiment under extreme conditions (Ferreira *et al*. 2007). Therefore, the objective of this study was to acquire the optimum condition on the enzymatic hydrolysis process by the BBD. This may be the first study on optimizing the A-LD interaction with enzyme loading and hydrolysis time. The optimization of the PEG concentration was also conducted to compare the performance of A-LD with PEG.

EXPERIMENTAL

Materials

The Numbu variety SSB was collected from the BIOTROP plantation (Bogor, Indonesia). According to our previous study (Solihat *et al*. 2017), the SSB had an acid-insoluble lignin (AIL) content of 24.59 ± 0.21%, an acid-soluble lignin (ASL) content of 0.66 ± 0.00%, a holocellulose content of 79.28 ± 0.76%, an α-cellulose content of 43.66 ± 2.39%, and a hemicellulose content of 35.63 ± 1.63% where AIL and ASL was analyzed according to Adney and Baker method (2008), holocellulose by Wise method (1946), α-cellulose by Rowell method (2005), and hemicellulose by subtracting the holocellulose and α-cellulose. The *A. mangium* kraft black liquor was obtained from PT TELPP, (South Sumatera, Indonesia). Hydrochloric acid (HCl), sodium hydroxide (NaOH), and acetic acid were purchased from Merck (Darmstadt, Germany). The cellulase (*Trichoderma reesei* ATCC 26921) with an enzyme activity of 42.3 FPU/mL, the polyethylene glycol diglycidylethers (PEDGE) M_n 500, and the polyethylene glycol (PEG) 4000 were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Methods

Preparation of the kraft pulp and A-LD

The details of the material preparation and pulping process of the SSB were explained in a previous study (Fatriasari *et al.* 2015). The SSB pulp was obtained from 250 g (dried weight) of SSB chips that were subjected to kraft pulping with 17% active alkali and 20% sulfidity for 2.5 h at 170 ℃. The two-step acid isolation procedure was used to isolate the lignin from the *A. mangium* black liquor, according to the method of Hermiati *et al*. (2017) with slight modification. One mol/L HCl was added to 100 g of black liquor with a pH of 12 until the pH reached 7, after which ethanol was added at a rate 4 times the volume of the HCl. The solution was kept at room temperature for 24 h. The ethanol was decanted, and the addition of HCl was continued until the solution reached a pH of 2. The solution was then filtered by technical filter paper. The lignin suspension was kept for 24 h, and the supernatant was decanted. The precipitated lignin was stored in a freezer at −18 °C for 24 h and filtered by a technical paper filter. The lignin powder was obtained after drying the precipitate at 45 °C for 24 h. The A-LD was synthesized according to the optimization condition by Fatriasari *et al.* (2020a). The lignin stock was prepared by dissolving 1 g of lignin in 50 mL NaOH (1 mol/L). Ten mL of lignin stock was added to 3 g of PEDGE M_n 500. Based on the previous study by Fatriasari *et al*. (2018), this molecular weight yielded a better RSY compared to when PEDGE with a higher M_n was used. The solution was heated at 60 °C for 1 h and the A-LD was formed after adding acetic acid until the pH reached 4 to stop the reaction.

Enzymatic hydrolysis and pretest experimental parameter

The enzymatic hydrolysis process was carried out in a WiseCube WIS-30R shaking

incubator (witeg Labortechnik GmbH, Wertheim, Germany). 0.2 g of SSB pulp was placed in a 100 mL Erlenmeyer flask. Two hundred µL of 2% (w/v) sodium azide, 4 mL of enzyme, and sodium citric buffer of 0.05 mol/L (pH 4.8) were added until total weight of the solution was 20 g. The different concentrations of enzyme that were added are listed in Table 1. The surfactant was subsequently added in the flask. Each flask was incubated at 50 °C and 150 rpm, according to the conditions in the BBD (Table 1).

Variations of enzyme loading, surfactant concentrations, and hydrolysis time were conducted for screening experimental parameters by using the one-factor at one time (OFAT) design. The screening experimental parameter was the first step to investigate the variables that affect the RSY as a response. Firstly, to study the effect of enzyme loading on the RSY, 0.2 g of pulp was added to 5% of A-LD. The used concentration of A-LD in this study is based on the authors' earlier findings (Fatriasari *et al*. 2018). The PEG 4000 2.5 g/100 g substrate was hydrolyzed using enzymes at concentrations of 10, 20, 30, 40, and 50 FPU/g. The incubation time was conducted for 72 h. The following boundary values were selected to evaluate the effect of the surfactant on the RSY: 0.2 g of pulp, 30 FPU/g of enzyme loading, 72 h of hydrolysis time, and 0.5 to 4.6 g/100 g of PEG 4000 as a substrate. Afterward, the effect of the hydrolysis time was conducted in variation times of 6 to 96 h with constant parameters: 0.2 g of pulp, 30 FPU/g of enzyme loading, 2.5 g/200 g of PEG substrate, and 5% of A-LD. Each test was measured in triplicate.

Reducing sugar analysis

The RSY in aliquot after the enzymatic hydrolysis process was analyzed *via* the dinitrosalicylic acid (DNS) method (Adney and Baker 2008). An ultraviolet (UV) Hitachi U-2001 spectrophotometer (Tokyo, Japan) at 540 nm wavelength was used to quantify the reducing sugar concentration (RSC). The RSY was determined by Eq. 1, and the total weight of the enzymatic hydrolysis was equal to 20 mL (Anita *et al.* 2020),

$$RSY\ (\%) = \frac{RSC \times 20}{ODW} \times \frac{PR}{100} \quad (1)$$

where *RSC* is in g/L, 20 is the total weight of enzymatic hydrolysis solution (g), *ODW* is oven dry weight of kraft pulp (g), and *PR* is the recovery of the pulp (%).

BBD with RSM

After the screening test, the relationship between the RSY and three process variables was investigated using the BBD. The number of the experiments in the BBD was defined as N = 2K(K-1) + C0, where K and C0 represent the number of level factorials and the number of central points. In this study, three level-factorials (K) such as the concentration of the surfactant, the enzyme loading, and the hydrolysis time were involved to investigate the influencing parameters to enhance the RSY and three central points (C0) were applied (Table 1) (Ferreira *et al*. 2007). Therefore, 15 run experiments were required. Table 1 shows the ranges and levels for the three factors in this study where the codes −1, 0, and +1 represent the low, central point, and high values. To identify the optimum point, Eq. 2 presents the mathematical relationship between the RSY as a response and three-independent variables (Pasma *et al*. 2013),

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^3 \quad (2)$$

where Y is the predicted response; X_1, X_2, and X_3 are independent variables; A_1, A_2, and A_3

are linear coefficients; A_{12}, A_{13}, and A_{23} are two interaction coefficients; and A_{11}, A_{22}, and A_{33} are quadratic coefficients.

Table 1. Box-Behnken Coded of the Three Factor Levels and Range for Enzymatic Hydrolysis using PEG 4000 and A-LD

Variable		Unit	Symbol	Level		
				-1	0	+1
Concentration	PEG 4000	g/100 g	X_1	2.5	3.5	4.5
	ALD	%		3	5	7
Enzyme loading		FPU/g	X_2	30	40	50
Hydrolysis time		Hour	X_3	48	72	96

RESULTS AND DISCUSSION

Screening Parameter Results

The results from the OFAT experiment, such as the effect of the enzyme loading, the surfactant dosage, and the hydrolysis time on the RSY are shown in Fig. 1. The OFAT method reduced the number of trials run and determined each factor effect to fix the other factors (Fatriasari *et al*. 2018). The effects of the enzyme loading on the RSY at 10 to 50 FPU/g intervals with and without the assistance of PEG 4000 (2.5 g/100 g) and A-LD (5%) are described in Fig. 1a. As the enzyme loading increased, the RSY also increased. Once the enzyme loading addition rate reached more than 40 FPU/g, there was no impact on the final sugar yield. This may be because there was no more altering of the velocity of the reaction from the enzyme in the substrate (Mussatto *et al*. 2008). Hence, the addition of 40 FPU/g was selected as an optimum condition for further experiments. In general, the RSY from enzymatic hydrolysis with the addition of A-LD was slightly higher than it would be with the addition of PEG 4000 in every bar of enzyme loading. The result is similar to a previous study that added EHL-PEG during the enzymatic hydrolysis process, where the glucose yield was enhanced by approximately 18% after PEG was added. This was attributed to EHL-PEG having a larger influence on minimizing the non-productive binding of the enzyme on lignin (Lin *et al*. 2015).

As shown in Fig. 1a and 1b, the surfactants effectively increased the RSY. This finding is similar to previous work done by Fatriasari *et al.* (2018), where 5% of surfactant addition increased RSY to 81.33%. Surfactant improved the digestibility in the enzymatic hydrolysis of the pulp by preventing non-productive enzyme adsorption and eventually maintained high enzyme activity during the process (Fatriasari *et al*. 2018). The effect of the surfactant in elevating the RSY has been confirmed by previous studies. Sipos *et al*. (2011) explained that adding PEG increased 3% of the RSY due to reducing unproductive absorption, decreasing protein adsorption, and increasing enzyme activity during enzymatic hydrolysis. Li *et al*. (2016) revealed that the surfactant sorbitan (80) monooleate can adsorb the presence of lignin in biomass substrates, enhancing the accessibility of enzymes during the hydrolysis enzymatic process. Another study found that surfactant concentration like PEG was the most important variable for the enzymatic hydrolysis of corn stover to release a high glucose yield (Eckard *et al*. 2011). In confirmation with the results of previous studies, the RSY in this study gradually increased as the surfactant dosage increased. This may have been an effect of the hydrophobic interaction of the surfactant (Eriksson *et al*. 2002). However, the RSY did not substantially increase after 2.5 g/100 g of PEG was added. Therefore, the concentration of 2.5 g/100 g PEG 4000 was

chosen as an optimum condition for further optimization. Figure 1c shows that the total amount of the RSY grew as the hydrolysis time increase, but after 72 h it decreased slightly. This may have been due to byproducts, such as hydroxymethyl furfural, from the dehydration of sugar (Daorattanachai *et al*. 2011). Thus, 72 h was decided as the optimum hydrolysis time for further investigation.

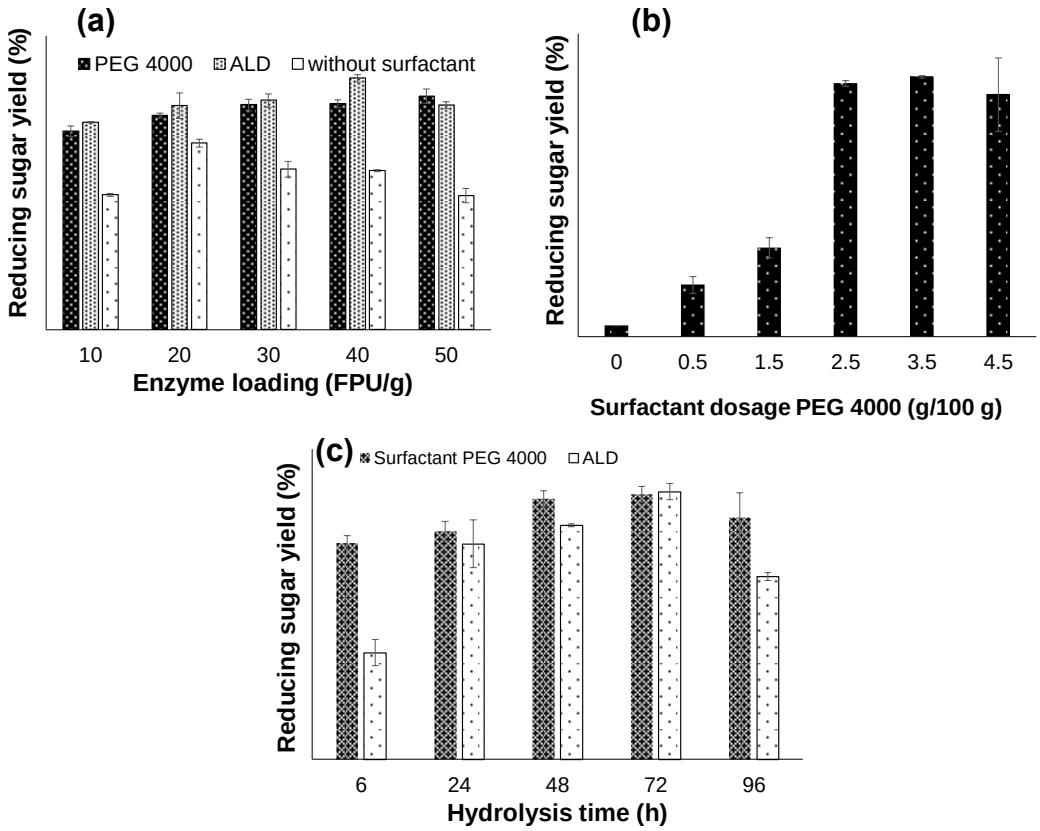

Fig. 1. The effect of (a) enzyme loading with and without the addition of PEG 4000 (2.5 g/ 100 g) and A-LD (5 %), (b) PEG 4000 concentration, and (c) hydrolysis time with surfactant-assisted at concentration 2.5 g/ 100 g PEG 4000 and 5 % A-LD on the RSY

Experimental Optimization Using BBD

A total run of the experiment details of the BBD is given in Table 2. The multiple regression analysis was calculated based on the RSY values in Table 2. The following equations represent a function of the predicted model for the RSY (%) from the enzymatic hydrolysis with the addition of the PEG 4000 (Eq. 3) and A-LD (Eq. 4) surfactants.

$$Y = 65.4 + 12.41X_1 - 0.678X_2 + 0.231X_3 - 2.639X_1^2 - 0.00899X_2^2 - 0.00488X_3^2 - 0.0535X_1X_2 + 0.1194X_1X_3 + 0.00407x_2X_3 \quad (3)$$

$$Y = -31.8 + 11.72X_1 + 0.999X_2 + 1.810X_3 - 0.447X_1^2 + 0.01144X_2^2 - 0.009612X_3^2 - 0.0369X_1X_2 - 0.0721X_1X_3 + 0.00319X_2X_3 \quad (4)$$

where Y is the RSY (%), X_1 is the concentration of the surfactant (g/100 g for PEG 4000 in Eq. 3 and % for A-LD in Eq. 4), X_2 is the enzyme loading (FPU/g), and X_3 is the hydrolysis time (h). The analyses of statistical significance were carried out using F test for analysis of variance (ANOVA) using Minitab 17 software (USA). If the p value was below 0.05, the model was significant. If the p value was above 0.05, the model was insignificant.

Table 2. Box-Behnken Design with RSM for the RSY of the SSB Pulp after Enzymatic Hydrolysis with the Addition PEG 4000 and A-LD

No	Concentration		Enzyme Loading (FPU/g)	Hydrolysis Time (hour)	RSY (%)	
	PEG 4000 (g/100 g)	A-LD (%)			PEG 4000	A-LD
1	2.5	3	40	48	85.69	76.84
2	3.5	5	30	48	85.64	83.49
3	3.5	5	50	48	85.33	81.53
4	4.5	7	40	48	75.76	86.91
5	2.5	3	30	72	82.10	86.91
6	2.5	3	50	72	87.42	93.95
7	3.5	5	40	72	91.00	91.19
8	3.5	5	40	72	87.44	93.73
9	3.5	5	40	72	89.73	97.42
10	4.5	7	30	72	88.95	89.89
11	4.5	7	50	72	92.13	93.98
12	2.5	3	40	96	86.39	93.71
13	3.5	5	30	96	87.67	91.92
14	3.5	5	50	96	91.27	93.02
15	4.5	7	40	96	87.92	89.93

RSY percentage is a factor to evaluate efficiency process of the BBD for the optimization of adding the A-LD and the PEG 4000 during enzymatic hydrolysis of SSB. A RSY between 75 and 97% was obtained from the following boundary parameters: PEG 4000 concentration of 2.5 to 4.5 g/100 g, A-LD concentration of 3 to 7%, enzyme loading between 30 and 50 FPU/g, and a hydrolysis time of 48 to 96 h (Table 2). Overall, the enzymatic hydrolysis with the addition of the A-LD released a higher RSY compared to the addition of the PEG.

The highest RSY (97.42%) was obtained under the following conditions: enzyme loading 40 FPU/g and hydrolysis time 72 h with A-LD 5% assisted. Moreover, an F-test and the determination of the regression coefficient (R^2) value were used to check the capability model. The results of the ANOVA test for the A-LD and PEG 4000 design in Table 3 show that both regression models were highly significant with $p < 0.05$. The F-values of the PEG 4000 and the A-LD were 8.03 and 30.3, respectively. A higher F-value means that the model is more statistically significant. The R^2 values for the PEG 4000 and A-LD were 0.7280 and 0.9100, respectively, which implies that both models are highly predictable because they can explain 72.8% and 91% variability in the response (Montgomery 2001). The R^2 value is in line with the p values of each variable where the p-value shows that the linear coefficients (x_1, x_2, and x_3) of the A-LD model were significant, and x_2 and x_3 of the PEG model were significant. In quadratic and two-way interactions coefficient, x_1^2, x_3^2, and x_1x_3 were significant for both surfactants. Overall, the p-values of the linear, quadratic, and two-way interactions of the variable that were used to explain the effect of three-level factors on the RSY were significant and suitable to predict the RSY according to the established parameters.

Table 3. ANOVA Model of the BBD for the Enzymatic Hydrolysis with the Addition of PEG 400 and A-LD

Source	Degree of Freedom	Sum of Squares		Mean Squares		F-Value		p-Value*	
		PEG 4000	A-LD	PEG 4000	A-LD	PEG 4000	A-LD	PEG 4000	A-LD
Model	11	503.494	1181.400	45.772	107.400	8.03	30.34	0.000*	0.000*
Blok	2	0.000	0.000	0.000	0.000	0.00	0.00	1.000	1.000
Linear	3	218.579	666.300	72.860	222.100	12.78	62.74	0.000*	0.000*
X_1	1	3.745	32.430	3.745	32.434	0.66	9.16	0.423	0.005*
X_2	1	52.127	39.550	52.127	39.552	9.15	11.17	0.005*	0.002*
X_3	1	162.708	594.310	162.708	594.314	28.55	167.88	0.000*	0.000*
Square	3	171.515	357.690	57.172	119.229	10.03	33.68	0.000*	0.000*
X_1^2	1	77.129	35.360	77.129	35.360	13.53	9.99	0.001*	0.003*
X_2^2	1	8.947	14.500	8.947	14.501	1.57	4.10	0.219	0.051
X_3^2	1	87.542	332.540	87.542	332.543	15.36	93.93	0.000*	0.000*
2-Way Interaction	3	113.399	157.420	37.800	52.472	6.63	14.82	0.001*	0.000*
X_1X_2	1	3.435	6.530	3.435	6.527	0.60	1.84	0.443	0.184
X_1X_3	1	98.499	143.870	98.499	143.867	17.28	40.64	0.000*	0.000*
X_2X_3	1	11.466	7.020	11.466	7.023	2.01	1.98	0.165	0.168
Error	33	188.077	116.830	5.699	3.540				
Lack-of-Fit	27	168.547	57.940	6.242	2.146	1.92	0.22	0.213#	0.997#
Pure Error	6	19.531	58.880	3.255	9.813				
Total	44	691.572	1298.203						

PEG 4000 : R^2 = 0.7280, Adjusted R^2 = 0.6374,
A-LD : R^2 = 0.9100, Adjusted R^2 = 0.8800
* p values below 0.05 were significant and p values above 0.05 were not significant
# Insignificant lack of fit means the model was highly predicted

The surface graph of the two interaction factors was drawn to visualize the correlation of the two independent variables and the one constant variable on the RSY. Figure 2a shows that the enzyme loading had a positive effect on the RSY. The RSY increased as the enzyme loading increased, although PEG 4000 had a quadratic effect on RSY. The RSY gradually increased from the lowest PEG 4000 concentration (2.5 g/100 g) and decreased after adding PEG 4000 at more than 3.9 g/100 g. Similar results were seen in a previous study in which increasing the surfactant concentration caused the RSY to decrease due to reverse micelles formation in the surfactant, which decreased the enzyme activity during the process (Pandey and Negi 2015). The strong interaction effect was also seen from the interaction of the PEG concentration and the hydrolysis time (Fig. 2b). The surface graph shows that the combination of the PEG 4000 and the hydrolysis time at the

lowest level had an RSY 84%, and it increased to 91% after 96 h of hydrolysis time and 3.6 g/100 g of PEG 4000 addition. The prolonged hydrolysis time elevated the RSY, while a PEG 4000 concentration higher than 3.6 g/100 g lowered the RSY. This may have been due to the rapid hydrolysis achieved at the middle surfactant concentration with long incubation time (Qi *et al*. 2009).

Low interaction was seen in the interaction of the enzyme loading and the hydrolysis time (Fig. 2c), such that the RSY was low at the low level of enzyme loading. At the lowest point of interaction, the RSY was less than 85% and it was elevated to 91% after the enzyme loading was increased to 50 FPU/g. In other words, the RSY did not significantly increase the enzyme loading increased unless the hydrolysis time was extended. This may be due to the increased enzyme activation to access the cellulosic substrate during the hydrolysis period (Jamaludin *et al*. 2013). According to the Multiple Response Prediction in the Minitab software, the maximum RSY (93.26%) was released under a PEG 4000 concentration of 4 g/100 g, an enzyme loading of 50 FPU/g, and a hydrolysis time of 92 h from this model.

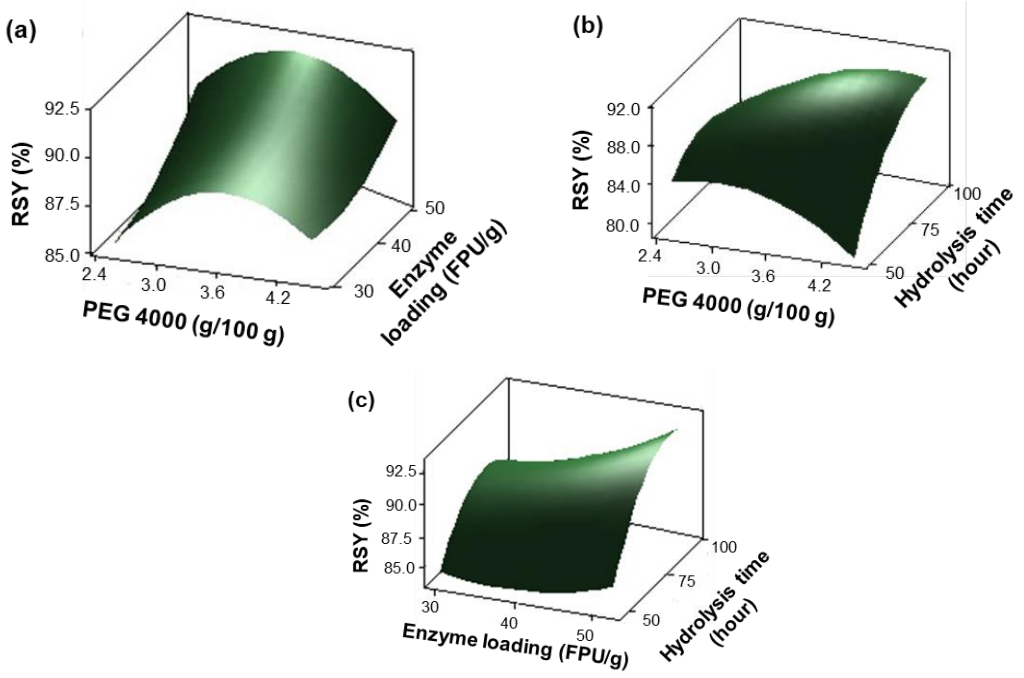

Fig. 2. The response surface graphs of the RSY showing two interaction factors on the enzymatic hydrolysis process with the addition of PEG 4000: (a) PEG 4000 concentration and enzyme loading, (b) PEG 4000 concentration and hydrolysis time, (c) Enzyme loading and hydrolysis time

The quadratic interaction of the A-LD concentration and the enzyme loading on the RSY can be seen in Fig. 3a. The trend was similar to the one shown in Fig. 2a, where the lowest point resulted in a RSY of 88%. By increasing the A-LD concentration to 5% with the enzyme loading at 49 FPU/g, the lighter green color was observed in the surface graph. The light green color correlated to the highest RSY (91%) in the model. The color gradually darkened again after it reached the optimum condition. On the other hand, the A-LD had a linear interaction effect with the hydrolysis time (Fig. 3b). However, the RSY declined by when the hydrolysis time was more than 90 h. This may have been caused by inhibitor production during the process that was not able to be controlled by adding surfactant even

at a high concentration (Qi and Volmer 2019). In this interaction, the lowest RSY of approximately 75% rapidly increased to 93% after the addition of A-LD 5% for 90 h of incubation time. Similar to Fig. 2c, weak interaction of enzyme loading and hydrolysis time was observed in Fig. 3c where the high RSY area in the model was achieved if the hydrolysis time increased. Of this model, the highest RSY (96.14%) was obtained after 89 h of hydrolysis time, 4% A-LD addition, and 50 FPU/g of enzyme loading based on the automatic calculation in the Minitab software. The maximum RSY of the A-LD assisted interaction was higher approximately 3% with a shorter hydrolysis time than with the PEG 4000 addition. This is in agreement with the screening single factor result in Fig. 1a.

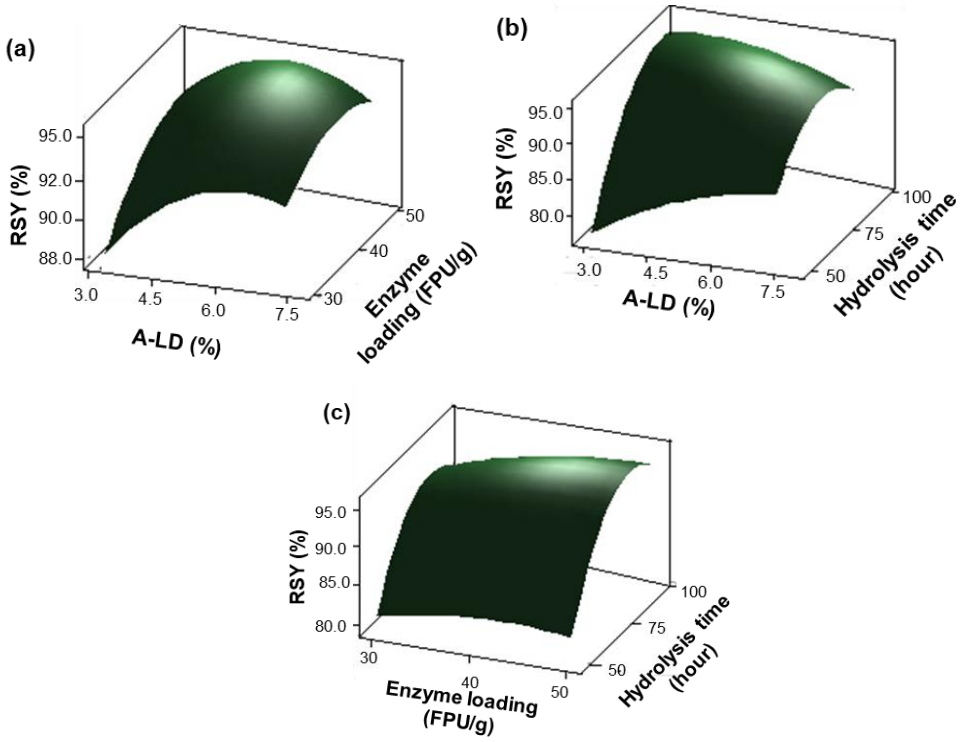

Fig. 3. The response surface graphs for the enzymatic hydrolysis with the impact of A-LD on the RSY of two interactions: (a) A-LD concentration and enzyme loading, (b) A-LD concentration and hydrolysis time, (c) Enzyme loading and hydrolysis time

The optimum condition was identified through the Minitab software. The optimum RSY with the PEG 4000 was achieved under the following conditions: PEG 4000 concentration of 4 g/100 g, 50 FPU/g of enzyme loading, and a hydrolysis time of 92 h. The optimum RSY with the A-LD was achieved under the following conditions: An A-LD concentration of 4%, 50 FPU/g of enzyme loading, and a hydrolysis time of 89 h. Furthermore, the pulp was hydrolyzed by using the optimum condition to validate the model. The comparison of the fitting and experimenting RSY results after the optimization for both the A-LD and PEG 4000 addition is shown in Table 4. The data in Table 4 were measured in triplicate. There was a strong agreement between the fitted and experimental values, which was illustrated by a difference of less than 4% in the experimental and theoretical values, which implies adequacy of the used model (Anita *et al.* 2020). Both RSY values from the experimental results were above the predicted values. This result was similar to the pretreatment of SSB of wet and dry bases (Guarneros-Flores *et al*. 2019). In

summary, the enzymatic hydrolysis assisted by the A-LD had a higher RSY with a shorter hydrolysis period (3 h) compared to that of the PEG 4000. Another study regarding the optimization of surfactant dosage on the RSY reported that a 95% RSY was obtained from wheat straw by alkaline peroxide pretreatment under the following conditions: 50 FPU/g of enzyme loading, 20 g/L of substrate concentration, 4 g/L of polysorbate (80) monooleate 80 concentration, and a hydrolysis time of 48 h. Through a surfactant optimization, extruded corn stover released a 98% glucose yield with 10.8 FPU/g of enzyme loading, 0.3 g of PEG, and 72 h of incubation time (Eckard *et al*. 2011). Compared to these previous studies, the RSY that was obtained in this study was slightly higher. Therefore, it can be inferred that surfactant-assisted optimization is substantial in the biomass hydrolysis process to elevate the RSY. This study also can be a complementary study to explore lignin derivatives as a surfactant for bioethanol production.

Table 4. The Predicted and Experimental RSY Values for the Enzymatic Hydrolysis After the Addition of PEG 4000 and A-LD at Optimum Conditions

Surfactant	Optimum Condition			RSY (%)		
	Concentration	Enzyme Loading (FPU/g)	Hydrolysis Time (hour)	Predicted	Observation	Residue
PEG 4000 (g/100 g)	4	50	92	93.26	94.44 ± 0.91	1.16
ALD (%)	4	50	89	96.14	99.45 ± 0.1	3.31

CONCLUSIONS

1. The response surface methodology (RSM) showed the surfactant affected the enzymatic hydrolysis of SSB kraft pulp to produce reducing sugar. The statistical analysis showed that both the enzyme loading, and the hydrolysis time significantly interacted with both surfactants, PEG 4000 and amphiphilic lignin derivative (A-LD).
2. The interactions had a significant effect on the obtained reducing sugar yield (RSY) values. Based on the regression equation obtained by RSM, the difference in the fitting and experimenting RSY values was less than 4%, with insignificant lack of fit that revealed the model was adequate.
3. After optimization, the presence of both PEG 4000 and A-LD as a surfactant successfully elevated the RSY values up to 94% and 99%, respectively. This implies that the surfactant had a crucial effect on elevating the RSY.
4. The A-LD assisted interaction produced a higher RSY compared to the addition of PEG 4000. The highest RSY was achieved under the following conditions: An A-LD concentration of 4%, 50 FPU/g of enzyme loading, and a hydrolysis time of 89 h.
5. It can be inferred that the proposed model of RSM with the BBD in this study is useful to determine the crucial factors on the enzymatic conversion process.

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