MJFAS MALAYSIAN JOURNAL OF FUNDAMENTAL AND APPLIED SCIENCES
PRINT ISSN: 2289-5981 | ONLINE ISSN: 2289-599X

RESEARCH ARTICLE

Two-stage pre-treatment of coffee pulp waste to optimize the reducing sugar production using enzymatic hydrolysis

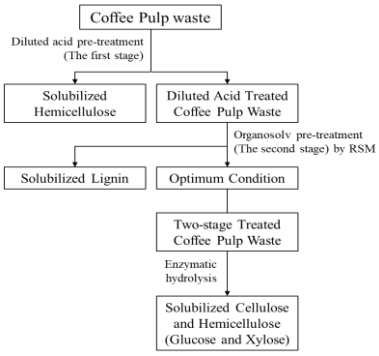
Tri Widjaja*, Ali Altway, Fibrillian Zata Lini, Toto Iswanto

Department of Chemical Engineering, Institut Teknologi Sepuluh Nopember, Sukolilo, Surabaya, Indonesia

* *Corresponding author: triw@chem-eng.its.ac.id*

Article history
Received 21 October 2018
Revised 13 February 2019
Accepted 12 September 2019
Published Online 3 December 2019

Graphical abstract



Abstract

Robusta coffee (*Coffee robusta L.*) pulp waste has been known for its high cellulose and hemicellulose content which potentially could be utilized as a source of a reducing sugar feedstock. Unfortunately, it contains inhibitors such as lignin, tannin, caffeine, and total polyphenols that can inhibit the enzymatic hydrolysis process. Therefore, coffee pulp waste needs pre-treatment prior to its utilization in reducing sugar production. To optimize the pre-treatment condition, the two-stage of pre-treatment process was carried out using 0.2 M sulfuric acid and then organosolv using ethanol. Subsequently, the optimization was done using Response Surface Methodology (RSM), 2^3 full factorial design, with the following input variables: ethanol concentration, temperature, and duration of pre-treatment. This study was subjected to determine the optimum conditions for organosolv pre-treatment which resulted in inhibitor removal and high concentration of reducing sugar. The hydrolysis process was carried out for 60 hours using a mixture of enzymes with and without the addition of Tween 80 as a surfactant. The result indicated that the lignin removal percentage increased from 0.85% (w/w) to 16.905% (w/w) towards the remaining lignin grams, with the change of crystallinity index of cellulose from 17.23% into 16.43%. The concentration of obtained reducing sugar with the addition of Tween 80 was 2.402 mg/ml, 1.6 times higher than that obtained without the addition of Tween 80.

Keywords: Central composite design, *Coffee robusta* L., enzymatic hydrolysis, reducing sugar, waste

INTRODUCTION

In Indonesia, the processing of coffee during 2011–2015 season yielded about 1.65 Mt of coffee pulp waste (*Coffee robusta* L.) [1]. According to the data of [2], the coffee production of that country in 2017 reached 463.775 ton. This waste has the potential to be converted to high-value products due to its high lignocellulosic content. The previous work was reported that coffee pulp waste contains 33.85% cellulose, 1.24% hemicellulose, 9.40% lignin, 0.81% caffeine, 0.81% total polyphenol, 1.61% tannin, and 52.28% miscellaneous [3]. So far, it has been utilized only as fertilizer, cattle feed, and compost. Unutilized coffee pulp waste in the environment will lead to a major problem in the soil and water ecosystem due to the released of its contents such as caffeine, free phenol, and tannins which are toxic for biological processes. Lignin covers the cellulose and hemicellulose causes a negative effect such as fiber-swelling inhibition (which alters the cellulose accumulation) which inhibits hemi-cellulosic enzyme activity [4]. Furthermore, caffeine needs to be eliminated before it is biologically or enzymatically hydrolyzed due to its anti-nutritional factor and toxic properties [5]. Moreover, the content of phenol also can inhibit the activity of cellulase in the hydrolysis process which is formed by the breakdown of lignin [6].

To increase the added value of coffee pulp waste, the attempt to convert it as reducing sugar feedstock can be chosen. Reducing sugar is the intermediate product obtained from biomass that can be converted into fuel by the fermentation process [7].

To obtain a high yield of reducing sugar, several factors must be taken into account such as biomass characteristics, pre-treatment conditions, and the use of enzyme formulations [8]. There are three methods of pre-treatment processes that are commonly used such as hydrothermal, acids, and organosolv. Hydrothermal pre-treatment is usually used due to the absence of chemical solution which potentially causes corrosion to the reactor. However, it is operated at high temperatures leading to the increase of the energy consumption and high operational cost [9]. On the other hand, the preferred method for acid pre-treatment was the use of dilute acid solution to minimizes the formation of inhibitor components (such as phenolic acids) and to reduces corrosion of the apparatus [10]. From the various pre-treatment methods, organosolv process is considered as the most promising for hydrolyzing lignin bonds and lignin-carbohydrate bonds, result in cellulose-rich solids residue [11] and dissolving lignin which will inhibit the metabolism of microorganisms. Commonly, a single way of pre-treatment could only partially change the material structure which still results in the inaccessible material by the enzyme during the hydrolysis process [12]. Mussato and Roberto [13] reported that the combinations of different treatment have been amply used to detoxify lignocellulosic hydrolyzates by removing lignin derivatives. They reported that the utilization of alkaline treatment (using CaOH) and then acid treatment (using H_2SO_4) could remove lignin derivatives to 95.4%. Brodeur *et al.* [12] found that the combination of acid pre-treatment and organosolv pre-treatment successfully increased the efficiency of delignification process. Moreover, 7.27 % (w/w) of lignin

removal was obtained from the phosphoric acid pre-treated bagasse which then increased to 11.42% (w/w) after organosolv pre-treatment using N-methyl morpholine N-oxide at 100 °C. Furthermore, Mesa *et al.* [15] showed that 0.85% (w/w) of lignin in sugar cane was successfully removed after sulfuric acid pre-treatment at 190 °C, and increased to 17.10 % (w/w) after organosolv pre-treatment using ethanol at 175 °C. Acid pre-treatment is capable to hydrolyze hemicellulose as well as the lignin structure in biomass. Thus, cellulose will have higher accessibility in the enzymatic hydrolysis process which will eventually increase the conversion efficiency [14,16].

In order to obtain reducing sugar from the coffee pulp waste, cellulose and hemicellulose need to be hydrolyzed. Several factors have negative effects in inhibiting enzymatic hydrolysis processes, such as lignin, acetyl group, and cellulose crystallinity [4]. In the enzymatic hydrolysis process, the use of cellulase enzymes is needed to convert cellulose into cellobiose by bound fractions such as endoglucanase and exoglycanase. While the cellobiose enzyme is used to convert cellobiose (as an intermediate fraction) to glucose by unbound fraction such as β-glucosidase. Meanwhile, the xylanase fraction is converted to xylose by using xylanase enzyme [17]. In this study, surfactant was used to optimize the enzyme hydrolysis. The addition of surfactant was subjected to increase the efficiency of the enzymatic hydrolysis process by reducing the surface tension between the two liquid phases [18]. The study by Li *et al.* [19] revealed that Tween 80 might relieve cellulase adsorption on some corn stover due to the adsorption of Tween 80 to lignin which occupied a part of the lignin hydrophobic surface in the corn stover. Furthermore, they pointed out that Tween 80 had a very high influence on xylanase adsorption and desorption on/from lignin rather than Polyethylene glycol (PEG).

Response surface methodology (RSM) is a mathematical and statistical method that has been widely used. In the study by Timung *et al.* [8], optimization was done on acid pre-treatment and hot water pre-treatment with various temperature, biomass weight, and pre-treatment time. Likewise in the study by Humaidah *et al.* [20], optimization with RSM was conducted to determine the optimum condition of ethanol fermentation from *Borassus flabellifer* in various temperatures and pH. Also, the study by Lini *et al.* [21] performed optimization on the enzymatic hydrolysis process with the variation of pH, temperature and hydrolysis time.

Based on the given explanation, the present work was conducted in two stages of pre-treatment. The first stage used an acid solvent (0.2 M sulfuric acid). The second stage used an organic solvent (ethanol) at 127 °C with the aid of 3% (w/w) sodium hydroxide as a catalyst. The use of organic solvents such as ethanol is safer because it has lower toxicity than other alcohol such as methanol. The lower temperature than the previous study by Mesa *et al.* [15] has been chosen to reduce more amorphous decline of cellulose and reduce the formation of by-products such as furfural and Hydroxymethylfurfural (HMF) by acid pre-treatment at high temperatures [22]. In addition, the use of sodium hydroxide as a catalyst during the pre-treatment of organosolv ethanol enhanced the selectivity of ethanol to lignin as well as the ability of delignification by ethanol. In this study, the effect of different operating conditions factors such as organic solvent concentration (ethanol), pre-treatment time, and temperature to achieve optimum conditions during pre-treatment process based on RSM of design of experiments (DOE) was also examined. Then the pre-treated solids would be hydrolyzed with the various enzyme combination. The use of a mixture of cellulase-cellobiose enzyme and mixture of cellulase -xylanase enzyme with and without Tween 80 the addition as surfactant on each enzyme mixture is expected to increase the enzymatic hydrolysis performance.

EXPERIMENTAL

Materials

Coffee pulp waste (*Coffee robusta L.*) was obtained from PTPN XII Bangelan, Malang, East Java, Indonesia. The waste was dried under the sun and then its size was reduced to 120 meshes.

Experimental procedure

1. Dilute-acid pre-treatment (The first stage)

One hundred grams of dried solid coffee pulp waste and diluted sulfuric acid (0.2M H_2SO_4) was introduced into 250 ml Erlenmeyer flask with the solid-liquid ratio of 1:5 w/w and autoclaved at 120 °C for 40 minutes. The obtained solid residue was filtered and washed using water until it reached a neutral pH. Then, it was dried in an oven at 40 °C until constant weight was achieved.

2. Organosolv pre-treatment (The second stage)

Ten grams of the dried solid sample obtained from the first stage of pre-treatment was treated using organosolv process with a solid-ethanol ratio of 1:7 w/w. 4N NaOH 3% (w/w dried sample). The reaction was performed in an autoclave by following operation condition as listed in Table 1. Subsequently, the solid residue was separated by filtration and washed using water until it reached neutral pH and dried in an oven at 40 °C until constant weight was achieved.

Experimental design

Optimization using Response Surface Methodology (RSM) was conducted by Minitab 16 statistical software (Minitab Inc., ITS Surabaya, Indonesia). 2^3 full factorial design central composite design method was done with 3 independent variables, namely pre-treatment duration, temperature, and ethanol concentration, as listed in Table 1. Lignin removal was set as the response to determine the optimum operating condition of the second stage pre-treatment.

Table 1 Range and experiment level towards the independent variables of the organosolv pre-treatment process.

Independent variable	Symbol	Range and level		
		-1	0	+1
Ethanol concentration (% v/v)	A	30	40	50
Pre-treatment duration (min.)	B	20	40	60
Temperature (°C)	C	100	110	120

3. Enzymatic hydrolysis

For the enzymatic hydrolysis, 1 gram of coffee pulp obtained from two-stage pre-treatment at the optimum condition was added to the mixed enzyme. The mixed enzyme was made by mixing pure cellulase enzymes from *Aspergillus niger* (enzyme activity: 0.533 U/g) and pure xylanase from *Trichoderma longibrachiatum* (enzyme activity: 0.625 U/g). In other variables, a mixture of pure cellulase enzymes from *T. reesei* (enzyme activity: 0.893 U/g) and pure cellobiose from *A. niger* (enzyme activity: 250 U/g) were used. All mixtures were diluted using 5.5 citrate buffer until 100 ml. Moreover, the variable which consist of only cellulase enzyme was made of enzyme from *T. reesei* with enzyme activity of 0.893 U/g. Tween 80 was added as a surfactant with the surfactant to solid sample ratio of 3:1 (g/g). The sample was incubated in an incubator shaker at 60 °C, 125 rpm for 48 hours. The variables without Tween 80 were set as controls.

Analytical procedures

Lignocellulose content analysis was done using the gravimetric method by Brodeur *et al.* [12]. Caffeine analysis was performed using spectrophotometry in the wavelength of 273 nm. Total phenolic and tannins analysis was carried out using *Folinciocalteu* method as described by Makkar *et al.* [24]. Crystalline cellulose examination was conducted using *X-Ray Diffraction* (XRD) PANalytical X-pert Pro Diffractometer. The value of its crystallinity index (CrI) was calculated using equation (1).

$$CrI = (I_{002} - I_{am})/I_{002} \times 100\% \quad (1)$$

Scanning electron microscopy (SEM) was used to capture the morphology of coffee pulp before and after pre-treatment. Images of samples were acquired typically at 7 kV and taken using HITACHI FLEXSEM 1000, the latest generation with Ultra Variable Pressure Detector.

Reducing sugar concentration analysis was carried out using *3,5-dinitrosalicylic acid* (DNS) method by Miller [25]. Analysis of glucose and xylose was conducted using high-performance liquid chromatography (HPLC).

RESULTS AND DISCUSSION

Effect of pre-treatment process on the chemical composition of coffee pulp waste

First of all, the physical pre-treatment was applied to the coffee pulp waste by the sun drying process to avoid decomposition in the coffee pulp waste. Then, the waste was milled to obtain the size of 120 meshes. This was to done to expand the contact surfaces between enzymes and coffee pulp waste, and thus increasing the amount of hydrolyzed substrate and the effectiveness of cellulose and hemicellulose degradation using the enzyme to produce glucose and xylose. According to the study of Han *et al.* [26], the chosen mesh size can increase the speed of enzymatic hydrolysis on grain straw by 4.7 times than that without milling. As for the smaller size than 160 meshes, the result of enzymatic hydrolysis did not show any significant difference.

1. First stage pre-treatment (using diluted H_2SO_4)

The pre-treatment of the first stage of the study was carried out using an 0.2 M H_2SO_4 (0.94% w/w) in an autoclave at 120 °C for 40 minutes. Acid solvent was used to improve the efficiency of the organosolv process by suppressing the furfural formation of the degradation of a pentose sugar (hemicellulose). While hemicellulose has a random amorphous structure with a complex chemical composition and can easily hydrolyzed into sugar at high temperature using acid, cellulose is more resistant to acid and high temperatures than hemicellulose. The xylose concentration will decrease with the increase of acid concentration (from 0.75% H_2SO_4 to 1% H_2SO_4) and temperature (from 140 to 200 °C). See Table 2.

2. Second stage pre-treatment (Organosolv using ethanol)

The second stage pre-treatment was performed using organic solvent (ethanol) with various of ethanol concentration (30, 40, 50% v/v), pre-treatment time (20, 40, 60 min), and pre-treatment temperature (100, 110, 120 °C).

The lower and upper limit values of the ethanol concentration were set at 30% and 50%, respectively.based on the previous works. Mesa *et al.* [15] reported that the increase in ethanol concentration from 10 to 30% caused an increase in glucose content of 1.35 g/l. Meanwhile, another work by Widjaja *et al.* [9] showed that the use of 50% ethanol concentration produced the biggest reducing sugar of 3.48 g/l. Determination of the lower and upper limits of the temperature was based on the limitation of the operating temperature on the autoclave (127 °C). This was interesting to investigate the optimum temperature under this range, in order to reduce energy requirements during the process. The lower and upper limit of the pre-treatment duration was also determined based on previous research by Mesa *et al.* [15].

The experimental design was conducted by following the recommendation by RSM as listed in Table 5. Ethanol as an organic solvent in the second stage pre-treatment process was used because it is a renewable solvent that is capable of producing high recovery solids, protects the cellulosic fraction, increases the porosity of residual solids, and recovers high-quality lignin fractions [27]. The addition of 3% (w/w of a dried sample) NaOH was able to increase glucose yield after enzymatic hydrolysis as reported by Zhao *et al.* [28] who showed an elevated glucose yield of 30.4% after enzymatic hydrolysis using 3% (w/w) NaOH concentration.

The chemical composition of the coffee pulp waste was shown in Table 2. In the present study, the hemicellulose and lignin removal after the second stage pre-treatment reached to 86.45% (w/w) and 14.89%, respectively. This result was in accordance with the study by Mesa *et al.* [15] who found that after organosolv pre-treatment, hemicellulose and lignin in the coffee pulp waste could be removed up to 87.5% and 17.1% (w/w), respectively. Brodeur *et al.* [12] have pretreated the sugarcane juice using 0.5% (w/w) phosphoric acid solvent in the first stage, then using an organic solvent of 50% w/w N-methyl morpholine N-oxide (NMMO) which revealed that the lignin was able to be removed by 11.42%.

From the explanation above, it can be concluded that using the two-stage of pre-treatment could increase the percentage of lignin removal.

Table 2 The comparison of chemical composition in the coffee pulp waste before and after pre-treatment.

Component	Content in native coffee pulp waste (%)	After dilute acid pre-treatment		After organosolv pre-treatment, at the optimum condition	
		Content (g)	% removal	Content (g)	% removal
Hot water soluble	40.86	38.79	5.06	24.81	39.27
Cellulose	33.24	51.65	35.62	52.32	50.33
Hemicelluloses	16.49	2.52	84.71	2.24	86.45
Lignin	6.82	6.76	0.85	5.81	14.89
Ash	2.49	0.23	90.80	0.19	92.41
Tannins	0.02	0.01	80.50	0.004	85.07
Polyphenol	0.02	0.01	39.21	0.01	50.23
Caffeine	0.06	0.03	43.78	0.02	61.29

Effect of pre-treatment process on the cellulose crystallinity index

Cellulose is a complex biopolymer consisting of amorphous and crystalline cellulose where in comparison, the amorphous is easier to be digested by enzymes than crystalline cellulose [29]. To characterize the cellulosic structures of the coffee pulp waste, X-Ray Diffraction analysis was carried out before and after two-stage pre-treatment at the optimum condition. The sample was scanned at 2θ from 5° to 50°. The results of the X-Ray Diffraction analysis for both samples are shown in Fig. 1.

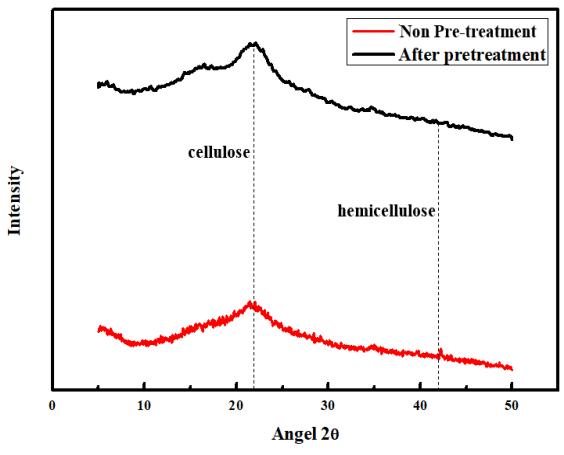


Fig. 1 Graph of X-Ray Diffraction analysis of coffee pulp waste before and after two-stage pre-treatment at the optimum condition.

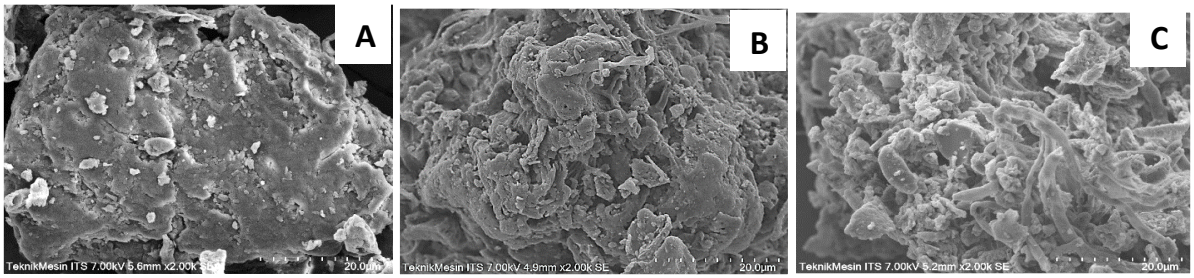


Fig. 2 SEM images of (A) untreated sample, (B) dilute acid pre-treated sample at the best variable and (C) Organosolv pre-treated sample at the optimum condition.

Fig. 1 shows that the crystalline fraction of cellulose (I_{002}) was at the maximum diffraction intensity of $2\theta = 22.5°$ (cellulose form most found in nature) and the amorphous cellulose fraction (I_{am}) was at the intensity of the minimum diffraction peak of $2\theta = 18.7°$ [10]. The difference was seen from the emerged peak where there was no more peak of $2\theta = 42.26°$ after the pre-treatment which was supposed to be the peak of hemicellulose [30]. This is in accordance with the results obtained where the hemicellulose level decreased from 16.490 % to 2.398 % after the organosolv pre-treatment.

To find out the value of the crystallinity index in both samples, the equation (1) was used. The calculated index is presented in Table 3.

Table 3 Crystallinity index of the coffee pulp waste.

No	Variable	I_{002}	I_{am}	CrI (%)
1	Non pre-treatment	366.042	302.970	17.231
2	Pre-treatment organosolv	520.210	434.756	16.427

The declining trend of cellulose values after organosolv pre-treatment showed that organosolv pre-treatment was able to alter cellulose structures to be more accessible to enzymes in the hydrolysis process. This was in line with studies conducted by Yoshida *et al.* [31] and Park *et al.* [29] who reported that glucose yields increased with the decrease in crystallinity caused by cellulosic amorphous hydrolysis which was faster than hydrolysis of cellulose crystallites. In addition, the decrease in the crystallinity value of the cellulose index was due to the use of a high pre-treatment temperature of 127 °C resulted in the disturbance of the amorphous structure of cellulose. Use of high-temperature during pre-treatment (greater than 100 °C) will result in a decrease of CrI value [16]. The difference was seen from the emerged peak where there was no more peak at $2\theta = 42.26°$ after the pre-treatment which reflected to be the peak of hemicellulose [30]. This is in accordance with the results, where the hemicellulose level decreased from 16.490% to 2.398% after the organosolv pre-treatment.

Morphological change after Two-Stage pre-treatment

The SEM of untreated, acid-pretreated, and organosolv-pretreated coffee pulp waste are shown in Fig. 2. When compared to the untreated coffee pulp, a compact surface was obtained. It was covered with a whole, smooth, and continuous surface. Conversely, the pretreated coffee pulp waste has a rough, broken face, and missing some parts of the outer surface. These conclude that pretreatment with acid-organosolv broke the pith cells and remove the external fibers (lignin). The removal of external fibers, in turn, led to increased surface area, which may have made the internal surface of lignocellulose (cellulose) was more exposed and possible to accessible enzymes.

Determination of the optimum conditions in organosolv pre-treatment using RSM

In this design, the input data analysis was done by the central composite design (CCD) method with three variables (2^3, resulted in the value of $\alpha = \sqrt{3}$ was equal to 1.732). That variables were ethanol concentration, temperature and pre-treatment duration. Based on the CCD, there were 19 runs that have been carried out in this study.

From the calculation in Minitab, the following equation can be used to calculate the predicted lignin removal at the optimum condition of organosolv pre-treatment.

$$\text{Lignin removal (g/g)} = 1.23 + 0.0852\,A + 0.0114\,B - 0.0395\,C - 0.001049\,A^2 - 0.000346\,B^2 + 0.000075\,C^2 - 0.000057\,AB + 0.000131\,AC + 0.000214\,BC \quad (2)$$

To find a significant effect of the independent variables, it was determined from the $P\text{-}_{value}$ which was smaller than 0.05. Table 4 shows that the significant independent variables were ethanol concentration, time and temperature, which means that by using ethanol, the independent variables as high concentrations of ethanol, time, and temperature (beyond the range of input variables included) were able to decrease the lignin content in the second stage pre-treatment process.

Table 4 Varian analysis (ANOVA) results from CCD experiment.

Source	DF	Sum of Squares	Mean Square	F-Value	P-Value
Model	9	0.940834	0.104537	16.31	0.000 s
Linear	3	0.508686	0.169562	26.45	0.000 s
A	1	0.253196	0.253196	39.50	0.000 s
B	1	0.137935	0.137935	39.50	0.000 s
C	1	0.117555	0.117555	21.52	0.001 s
Square	3	0.415054	0.138351	18.34	0.002 s
A^2	1	0.163894	0.163894	21.58	0.000 s
B^2	1	0.284890	0.284890	25.27	0.001 s
C^2	1	0.000835	0.000835	44.44	0.000 s
2-Way Interaction	3	0.017094	0.005698	0.13	0.726 ns
AB	1	0.001052	0.001052	0.89	0.483 ns
AC	1	0.001383	0.001383	0.16	0.695 ns
BC	1	0.014660	0.014660	0.22	0.653 ns
Error	9	0.057697	0.006411	2.29	0.165 ns
Pure Error	4	0.000005	0.000001		
Total	18	0.998531			

$R^2 = 0.942$ (s = significant ($P < 0.05$); ns = not significant ($P > 0.05$))

Fig. 3 shows the response surface and contour as the effect of ethanol concentration, temperature, and pre-treatment time on lignin removal. From Fig. 3A, the optimum condition was obtained in 51.29% (v/v) of ethanol concentration and 45 min of pre-treatment time to result in the lignin removal of 0.88 g/g. Meanwhile, the interactions between two variables indicated an insignificant effect, as shown in the response surface between pre-treatment duration and temperature (Fig. 3B and 3C). This means that the optimum condition for those variables could not be obtained in the investigated range.

The optimum condition obtained in the present study was 127 °C which able to save energy and to lower equipment costs compared to study conducted by Mesa *et al.* [14] who obtained the best organosolv pre-treatment process used 45% ethanol concentration at 185 °C for 60 minutes using a semi-pilot reactor.

The accuracy of the experimental results with the model can be seen from the R-square value. From Fig. 4, the red line shows the linear regression from the predicted and the experimental value. In this study, a fairly high R-square value of 94.22% was obtained. In addition, in Table 5, the predicted lignin removal gave a small error (0.058) to determine the observed lignin removal.

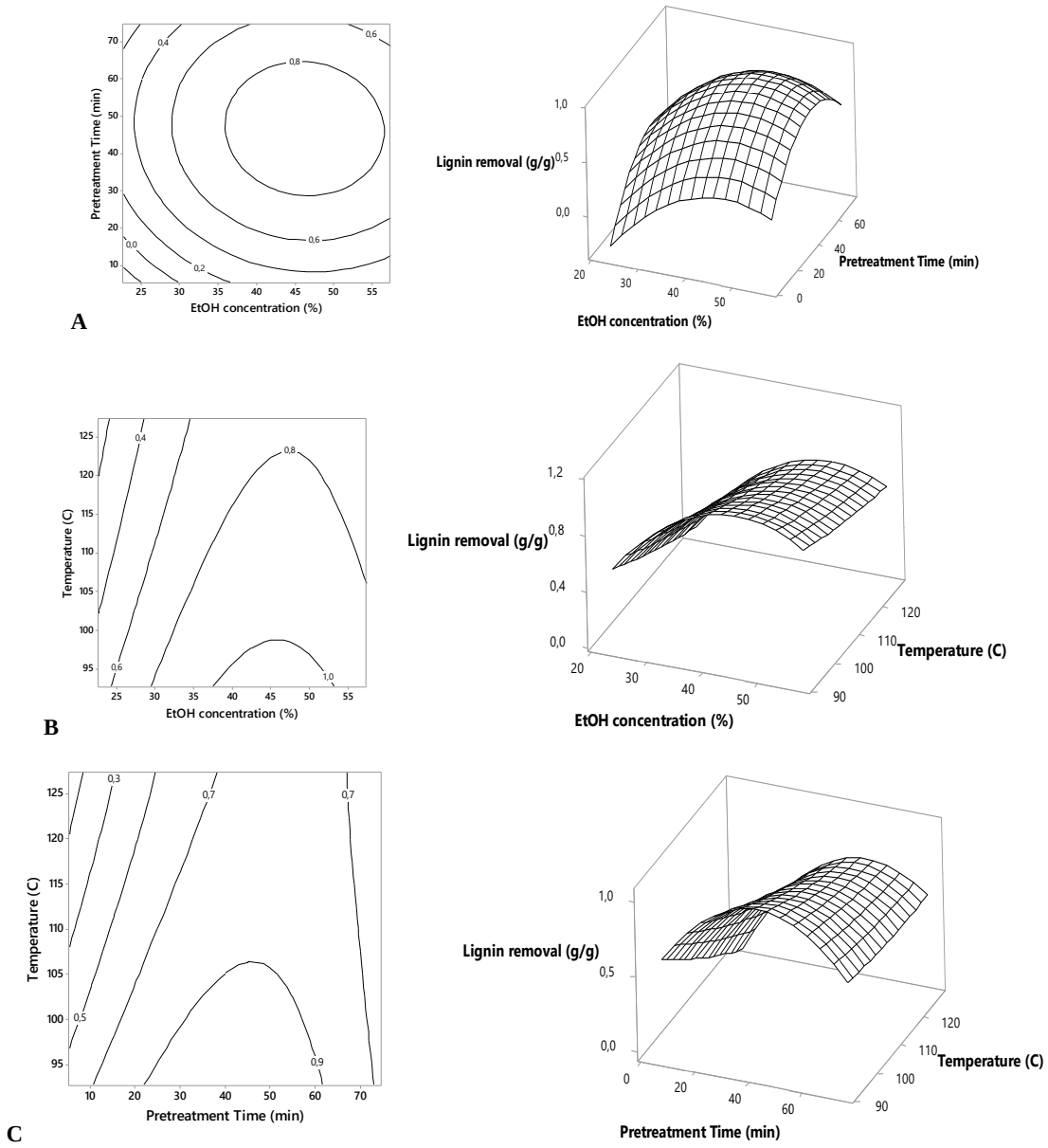


Fig. 3 Graphs of surface response and contour.

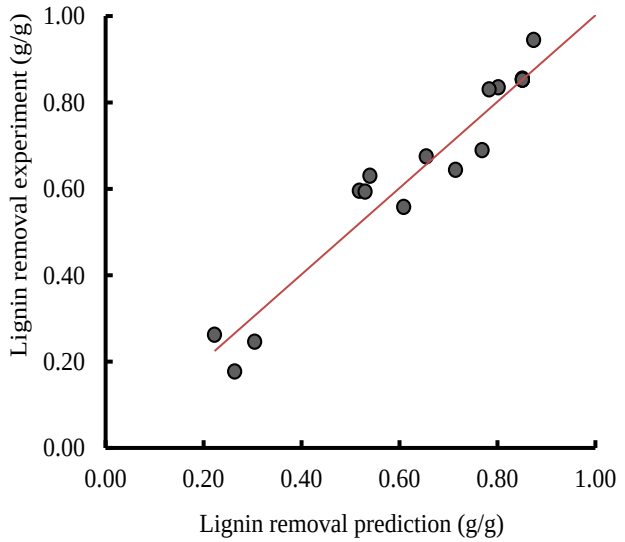


Fig. 4 Plots of Lignin removal value from the experiment vs prediction.

Table 5 Lignin removal resulted in the experiments and model.

Run	A	B	C	Lignin removal experiment (g/g)	Lignin removal prediction (g/g)
1	50	60	120	0.835	0.802
2	40	40	127.32	0.644	0.714
3	40	40	110	0.854	0.851
4	30	20	100	0.595	0.518
5	30	20	120	0.262	0.223
6	30	60	100	0.675	0.655
7	40	5.36	110	0.177	0.264
8	50	60	100	0.944	0.874
9	40	40	92.68	0.966	1.033
10	57.32	40	110	0.689	0.769
11	40	40	110	0.853	0.851
12	50	20	120	0.630	0.540
13	40	40	110	0.854	0.851
14	40	74.64	110	0.558	0.609
15	50	20	100	0.830	0.783
16	40	40	110	0.852	0.851
17	40	40	110	0.852	0.851
18	22.68	40	110	0.246	0.304
19	30	60	120	0.593	0.530

Effect of enzymatic mixed variance and surfactant on reducing sugar yields

Enzymatic hydrolysis was performed by calculating the enzyme activity to determine the needed amount of cellulase, xylanase, and cellubiase enzyme.

Table 6 The obtained reducing sugar from the hydrolysis of coffee pulp waste at the end of 48 h.

Varian	Reducing sugar concentration (mg/mL)	Reducing sugar yield (g/g)
Cellulase + Xylanase + Tween 80	2.349	0.016
Cellulase + Xylanase	1.308	0.009
Cellulase + Cellobiase + Tween 80	1.637	0.011
Cellulase + Cellobiase	0.199	0.001
Cellulase + Tween 80	1.451	0.009
Cellulase	0.181	0.001

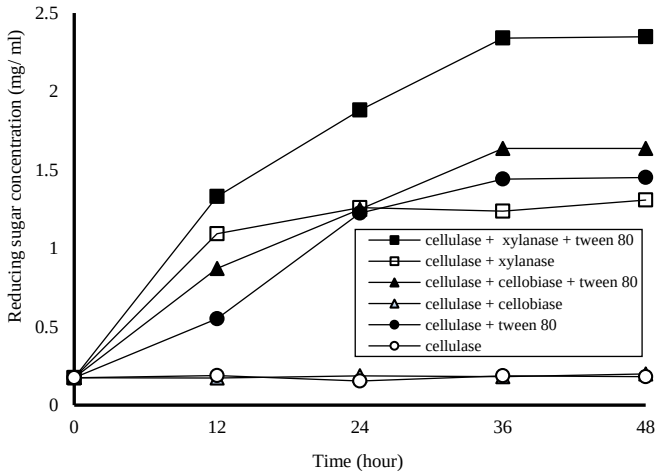


Fig. 5 Obtained reducing sugar concentration during the hydrolysis process.

Fig. 5 reveals that the hydrolysis using a mixture of cellulase and xylanase enzymes with Tween 80 had the tendency to produce higher reducing sugar concentrations rather than using the mixture of cellulase and cellobiose enzymes with Tween 80. This was caused by the presence of glucose as the main product produced in this enzymatic hydrolysis, had formed a little xylose which subsequently converted by xylanase. In addition, there was no inhibition of β-glucosidase in enzymatic hydrolysis. Alancer *et al.* [32] reported that inhibition of β-glucosidase did not occur because the yield of cellulose obtained was about 90% after 48-hour pre-treatment with acid solvents and surfactants. The failure of the addition of cellobiose to increase hydrolysis was also reported by Chen *et al.* [33]. They reported that the concentration of cellobiase retained at low concentrations experiencing a rapid increase of activity. Thus, inhibition of feedback caused by cellobiose accumulation was greatly reduced, resulting in a decrease in sugar concentration due to the poor performance of cellobiase.

Compared to the control, the hydrolysis yield was increased up to 44.33 % at 48 hours as the effect of Tween 80 addition. Alencer *et al.* [32] indicated that the usage of Tween 80 can increase 50% concentration of reducing sugar. There are several reasons for the addition of surfactants into enzymatic hydrolysis processes, involve; (1) surfactants can increase stability and prevent denaturation of enzymes, (2) surfactants may affect substrate structures and make substrates more accessible during hydrolysis by reducing surface tension, and (3) surfactants may affect substrate-enzyme relation which will increase the effectiveness of cellulose conversion into reducing sugars. Based on the research by Zhou *et al.* [34], the addition of non-ionic surfactants to the hydrolysis process will produce a significant result as long as it did not more than 5 g. A significant effect could not be observed due to the saturated solubility of the surfactant.

The increased concentrations of reducing sugars also occurred in the use of enzyme mixtures using cellobiose enzyme compared with the absence of enzyme mixtures. In this study, an increase of 11.7% was obtained. While the study by [35] showed that the use of cellobiose could increase the reducing sugar concentration to 18.9%. However, the increase was much lower than that with the use of a mixture of xylanase enzymes that enabled to increase concentrations of reducing sugars up to 34.7%. Meanwhile, [36] showed that the use of xylanase can increase the obtained reducing sugar to 35%. The results of HPLC Test of coffee pulp waste hydrolysate are listed in Table 7.

Table 7 The result of HPLC analysis on the hydrolysate.

Sample	Parameter	Result (% b/v)
Before pre-treatment	Glucose	0.00
	Xylose	0.00
After Pre-treatment	Glucose	1.30 ± 0.12
	Xylose	0.36 ± 0.03

Table 7 shows that glucose concentration obtained from cellulose conversion had a higher concentration than xylose concentration obtained from the conversion of coffee pulp waste hemicellulose. This condition supports the results of the gravimetric analysis which the hemicellulose concentration after pre-treatment decreased (up to 2.209%), less than cellulose (12.378%). Meanwhile, the hydrolysate of raw coffee pulp waste without pre-treatment did not produce a remarkable amount of glucose or xylose. This was due to the contents of cellulose and hemicellulose which were still bound by lignin, could be accessed by the enzyme. Therefore, it could not be converted into glucose and xylose.

CONCLUSION

The combination of acid pre-treatment and organosolv pre-treatment has obtained the optimum conditions of 44.88 min, 127 °C and 51.29% (v/v) of ethanol which revealed an efficient technique to hydrolyze robusta coffee pulp waste into enzymatic reducing sugar. With the decrease of crystallinity index from 17.231 % to 16.427 % caused the increasing of the amorphous content of cellulose. The developed method in this study has resulted in the total lignin removal of 16.905 % (w/w) and the total hemicellulose removal of 87.5 % (w/w). It led to easier access of enzyme towards the substrate's amorphous cellulose. The highest yield of reducing sugar was 0.016, obtained from hydrolysis using a mixture of cellulase and xylanase enzymes with the addition of Tween 80. The hydrolysis using a mixture of cellulase and xylanase enzymes with Tween 80 had the tendency to produce higher reducing sugar concentrations rather than using the mixture of cellulase and cellobiose enzymes with Tween 80.

ACKNOWLEDGMENT

The authors would like to express their gratitude to LPPM of ITS and to all members of Biochemical Technology Laboratory, Chemical Engineering ITS for their endless support.

REFERENCES

[1] Murthy, P. S., Naidu, M. M. 2012. Sustainable management of coffee industry by-products and value addition: A review. *Resour. Conserv. Recycl*. 66, 45–58.

[2] Widjaja, T., Iswanto, T., Altway, A., Shovitri, M., Juliastuti, S. R. 2017. Methane production from coffee pulp by microorganism of rumen fluid and cow dung in co-digestion. *Chem. Eng. Trans*. 56, 1465–1470.

[3] Directorate General of Estate Crops. 2016. *Statistik perkebunan Indonesia: 2015-2017*. Retrieved from kopiStatistik perkebunan indonesia/tree crop estate statistic of indonesia/2015-2017/Kopi/Coffee. Jakarta

[4] Eckard, A. D., Muthukumarappan, K., Gibbons, W. 2013. A review of the role of amphiphiles in biomass to ethanol conversion. *Appl. Sci.* 3, 396–419.

[5] Bi, W., Zhou, J., Row, K. H. 2011. Decaffeination of coffee bean waste by solid-liquid extraction. *Korean J. Chem. Eng*. 28, 221–224.

[6] Sun, S., Sun, S., Cao, X., Sun, R. 2015. The role of pretreatment in

improving the enzymatic hydrolysis of lignocellulosic materials. *Bioresour. Technol.*199, 49–58.

[7] Liang, J., Chen, X., Wang, L., Wei, X., Wang, H., Lu, S., Li, Y. 2017. Subcritical carbon dioxide-water hydrolysis of sugarcane bagasse pith for reducing sugars production. *Bioresour. Technol*. 228, 147–155.

[8] Timung, R., Mohan, M., Chilukoti, B., Sasmal, S., Banerjee, T., Goud, V. V. 2015. Optimization of dilute acid and hot water pretreatment of different lignocellulosic biomass: A comparative study. *Bioma. Bioener.* 81, 9–18.

[9] Widjaja, T., Altway, A., Nurkhamidah, S., Edahwati, L., Lini, F. Z., Oktafia, F. 2016. The effect of pretreatment and variety of microorganisms to the production of ethanol from coffee pulp. *Arpn J. Eng. Appl. Sci*. 11, 1056–1060.

[10] Karapatsia, A., Pappas, I., Penloglou, G., Kotrotsiou, O., Kiparissides, C. 2017. Optimization of dilute acid pretreatment and enzymatic hydrolysis of phalaris aquatica l. lignocellulosic biomass in batch and fed-batch processes. *Bioener. Res*. 10, 225–236.

[11] Andrić, P., Meyer, A. S., Jensen, P. A., Dam-Johansen, K. 2010. Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis: i. significance and mechanism of cellobiose and glucose inhibition on cellulolytic enzymes. *Biotechnol. Adv*. 28, 308–324.

[12] Brodeur, G., Telotte, J., Stickel, J. J., Ramakrishnan, S. 2016. Two-stage dilute-acid and organic-solvent lignocellulosic pretreatment for enhanced bioprocessing. *Bioresour. Technol*. 220, 621–628.

[13] Mussatto, S. I., Roberto, I. C. 2004. Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: A review. *Bioresour. Technol*. 93, 1–10.

[14] Mesa, L., López, N., Cara, C., Castro, E., González, E., Mussatto, S. I. 2016. Techno-economic evaluation of strategies based on two steps organosolv pretreatment and enzymatic hydrolysis of sugarcane bagasse for ethanol production. *Renew. Energy.* 86, 270–279.

[15] Mesa, L., González, E., Cara, C., González, M., Castro, E., Mussatto, S. I. 2011. The effect of organosolv pretreatment variables on enzymatic hydrolysis of sugarcane bagasse. *Chem. Eng. J*. 168, 1157–1162.

[16] Timung, R., Deshavath, N. N., Goud, V. V, Dasu, V. V. 2016. Effect of subsequent dilute acid and enzymatic hydrolysis on reducing sugar production from sugarcane bagasse and spent citronella biomass. *J. Ener*. 2016, 1-12.

[17] Fenila, F., Yogendra, S. 2016. Optimal control of enzymatic hydrolysis of lignocellulosic biomass. *Resour. Technol.* 2, S96–S104.

[18] Chang, K., Chen, X., Han, Y., Wang, X., Potprommanee, L., Ning, X., Liu, J., Sun, J., Peng, Y., Sun, S., Lin, Y. 2016. Synergistic effects of surfactant-assisted ionic liquid pretreatment rice straw. *Bioresour. Technol*. 214, 371–375.

[19] Li, Y., Sun, Z., Ge, X., Zhang, J. 2016. Effects of lignin and surfactant on adsorption and hydrolysis of cellulases on cellulose. *Biotechnol. Biofuels.* 9, 1-9.

[20] Humaidah, N., Widjaja, T., Budisetyowati, N., Amirah, H. 2017. Comparative study of microorganism effect on the optimization of ethanol production from palmyra sap (borassus flabellifer) using response surface methodology. *Chem. Eng. Trans*. 56, 1789–1794.

[21] Lini, F. Z., Widjaja, T., Hendrianie, N., Altway, A., Nurkhamidah, S., Tansil, Y. 2018. The effect of organosolv pretreatment on optimization of hydrolysis process to produce the reducing sugar. *Matec Web Conf.* 10, 1056–1060.

[22] Avci, A., Saha, B. C., Kennedy, G. J., Cotta, M. A. 2013. Dilute sulfuric acid pretreatment of corn stover for enzymatic hydrolysis and efficient ethanol production by recombinant escherichia coli FBR5 without detoxification. *Bioresour. Technol*. 142, 312–319.

[23] Datta, R. 1981. Acidogenic fermentation of lignocellulose–acid yield and conversion of components. *Biotechnol. Bioeng*. 23, 2167–2170.

[24] Makkar, H. P. S., Hagerman, A., Hervey, I. M. 2000. Quantification of tannins in tree and shrub foliage. Joint FAO/IAEA Division of Nuclear Tehniques Infood And Agriculture, Vienna.

[25] Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31, 426–428.

[26] Han, L., Feng, J., Zhang, S., Ma, Z., Wang, Y., Zhang, X., 2012. Alkali pretreated of wheat straw and its enzymatic hydrolysis. *Braz. J. Microbiol*. 43, 53–61.

[27] Wildschut, J., Smit, A. T., Reith, J. H., Huijgen, W. J. J. 2013. Ethanol-based organosolv fractionation of wheat straw for the production of lignin and enzymatically digestible cellulose. *Bioresour. Technol*. 135, 58–66.

[28] Zhao, X., Cheng, K., Liu, D. 2009. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. *Appl. Microbiol. Biotechnol*. 82, 815–827.

[29] Park, S., Baker, J. O., Himmel, M. E., Parilla, P. A, Johnson, D. K. 2010. Cellulose crystallinity index: Measurement techniques and their impact on interpreting cellulase performance. *Biotechnol. Biofuels.* 3, 1–10.

[30] Hamada, Y., Yoshida, K., Asai, R., Hayase, S., Nokami, T., Izumi, S., Itoh, T. 2013. A possible means of realizing a sacrifice-free three component separation of lignocellulose from wood biomass using an amino acid ionic liquid. *Green Chem*. 15, 1863.

[31] Yoshida, M., Liu, Y., Uchida, S., Kawarada, K., Ichinose, H., Kaneko, S., Fukuda, K. 2014. Effects of cellulose crystallinity, hemicellulose, and lignin on the enzymatic hydrolysis of miscanthus sinensis to monosaccharides. *Biosci. Biotechnol. Biochem.* 72, 3. 805–810.

[32] Alencar, B. R. A., Rocha, J. M. T. S., Rocha, G. J. M., Gouveia, E. R. 2017. Effect of tween-80 addition in dilute acid pretreatment of waste office paper on enzymatic hydrolysis for bioethanol production by SHF and SSF processes. *Cellul. Chem. Technol*. 51, 121–126.

[33] Chen, M., Xia, L., Xue, P. 2007. Enzymatic hydrolysis of corncob and ethanol production from cellulosic hydrolysate. *Int. Biodeterior. Biodegrad*. 59, 85–89.

[34] Zhou, Y., Chen, H., Qi, F., Zhao, X., Liu, D. 2015. Non-ionic surfactants do not consistently improve the enzymatic hydrolysis of pure cellulose. *Bioresour. Technol*. 182, 136–143.

[35] Chen, M., Zhao, J., Xia, L. 2008. Enzymatic hydrolysis of maize straw polysaccharides for the production of reducing sugars. *Carbohydr. Polym*. 71, 411–415.

[36] Tabka, M. G., Herpoël-Gimbert, I., Monod, F., Asther, M., Sigoillot, J. C. 2006. Enzymatic Saccharification of wheat straw for bioethanol production by a combined cellulase xylanase and feruloyl esterase treatment. *Enzyme Microb. Technol.* 39, 897–902.