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Abstract. This study has successfully investigated the effect of microorganisms, enzymes, and surfactants mixture to produce a reducing sugar from Coffee pulp waste. The experiment consisted of microbial pretreatment to reduce lignin content followed by comparing the hydrolysis by enzymes and microorganism using a surfactant and without surfactant to get a higher yield of reducing sugar. Pretreatment was conducted by mixture of *Bacillus subtilis* (BS) with *Trichoderma reesei* (TR) in the ratio of 2:1 (v/v) and *Aspergillus niger* (AN) with TR in the ratio of 1:1 (v/v). BS-TR mixture increased the cellulose content to 10.939 % and decreased the lignin and pectin content to 71.261 % and 55.046 %, respectively. Whereas, AN:TR mixture increased the cellulose content to 12.572 % and decreased the lignin and pectin content to 69.941 % and 52.294 %, respectively. Afterward, the result of enzymatic hydrolysis with 3 g of Tween 80 and biological hydrolysis showed increasing of reducing sugar yield of 0.5831 and 0.0341 %, respectively. Further investigation was described as the crystallinity index and the fructose, glucose, and sucrose contents. The addition of both PEG 4000 and Tween 80 as a surfactant in the enzymatic hydrolysis process could significantly increase the concentration of reducing sugar.

1. Introduction

Coffee is the second largest trading commodity in the world including in Indonesia which has a quite high productivity of 739.005 tons in 2015, harvested from the productive coffee plant area of 1,254,382 ha managed by people, government and private sector [1]. Hence, an enormous amount of Coffee Pulp Waste (CPW) was generated, but still without optimized utilization yet and low economic value. Farmers frequently threw away near plantation area or sold the waste at a meager price for cattle feed and compost, where in fact the waste can be utilized to produce more valuable products such as reducing sugar or methane production for biogas [2].

CPW contains 63 % of cellulose, 2.3 % of hemicellulose, 17 % of lignin, 11.5 % of protein, 1.8-8.56 % of tannin, 6.5 % of pectin, and 1.5 % of caffeine [3]. The presence of lignin, pectin, tannin, and caffeine in the CPW can inhibit reducing sugar formation, so it must be eliminated. However, the complex structure of lignin, pectin, hemicellulose, and cellulose contents in the CPW is formed by the covalent bonds, the intermolecular bonds, and the van der Waals force which is hardly broken down by hydrolysis process and also insoluble in the water [4]. Therefore, the pretreatment process is highly recommended.

Biological pretreatment presents a promising approach to convert lignocellulosic materials into reducing sugar, where it is safer and environmentally friendly. It also does not require substantial energy to degrade lignin from lignocellulosic biomass. Several types of microorganism were reported to degrade lignin and pectin. *Aspergillus niger* can effectively degrade lignin and pectin, *Bacillus subtilis* can degrade pectin, and *Trichoderma reesei* can degrade lignin [5]. In another study, *Aspergillus* and *Penicillium* fungus was known to have a good degradation ability of caffeine [6].
The aims of this study are reducing lignin and pectin level in CPW using *Aspergillus niger, Bacillus subtilis,* and *Trichoderma reesei* to obtain more natural accessible cellulose and hemicellulose; investigating the effect of the enzymatic hydrolysis from pretrated CPW using pure cellulase and xylanase enzymes and culture of *Aspergillus niger* and *Trichoderma viride* on the production of reducing sugar. The selection of the enzymatic hydrolysis method was based on the previous research which states that the enzymatic hydrolysis can produce high pure glucose yield with a specific process, can be carried out in low operating temperature, has low energy consumption and does not cause corrosion [7]. In the enzymatic hydrolysis process, PEG 4000 and Tween 80 as the surfactants was added which are useful for enlarging the conversion value of the biomass substrate and preventing deactivation of cellulase enzymes [8]. The effects of those surfactants on reducing sugar yield was also investigated in the present study.

2. Methods

2.1. Preparation of coffee pulp waste

Coffee pulp waste was obtained from Bangelan, East Java Indonesia. It was dried under the sunlight for approximately 7 days. Then the CPW was mashed using a grinding machine and sieved to get 120 meshes. The smaller substrate particles help the hydrolysis process to make the contact surface between the substrate and the enzymes larger and the lignocellulose content will be easier to be degraded. Before used, the CPW was dried using the oven at 105 ^{o}C to reduce moisture content.

2.2. Biological Pretreatment process of coffee pulp

Aspergillus niger, *Bacillus subtilis*, *Trichoderma viride*, and *Trichoderma reesei* were obtained from the Laboratory of Microbiology Engineering, Chemical Engineering Department FTI-ITS. One loop of each microbs was inoculated to 100 ml of sterilized nutrient broth and incubated for 96 h at 30 °C, and 1 ml of the inoculum sample was introduced into the test tube. The sample was diluted until 10 ml and counting chamber analysis method was done to find the log phase of the microorganism.
In the biological pretreatment, the CPW and water was mixed with ratio of 1:4. Thirty milliliter of the mixture of AN and TR with 1:1 (v/v) ratio; BS and TR with 2:1 (v/v) ratio were inoculated into the CPW solution and incubated at 35 °C and pH 5 for 4 d. The ratio between microorganism and CPW was 30:100 (ml/g). The obtained CPW after this pretreatment was used in the next step, the hydrolysis process.

2.3. Hydrolysis

The enzymatic hydrolysis process using cellulose and xylanase enzyme was performed by weighing 1 g of the pretreated CPW and put into an Erlenmeyer flask. 20,552 ml of cellulase and 14,524 ml of xylanase were added into the flask and then heated until 40 °C. Afterward, 0,1 M of citrate buffer with pH 5,5 was added into the enzyme-CPW solution until the total volume reached 30 ml. Then, the surfactant was added from 1 to 3 g. The sample was incubated using incubator shaker at 35 °C for 16 h.
The biological hydrolysis process using microorganism was carried out by weighing 1 g of pretreated CPW and introduced it into the Erlenmeyer flask. Three milliliter of the mixture of *Aspergillus niger* and *Trichoderma viride* with various ratio of AN:TV (1:1, 1:2, and 2:1) were inoculated into the flask and 0,1 M citrate buffer solution pH of 5,5 was added until the total volume reached 30 ml. Then, that flask was heated slowly until 30 °C. The surfactan was added into pretreated CPW solution in ratio 1 g per 100 g of CPW. The flask was incubated in the same condition above. Pretreated CPW and 0,1 M citrate buffer solution pH of 5,5 (total volume: 30 ml) was used as control.

2.4. Analysis

Gravimetric method by [9] was used to analyze cellulose, hemicellulose and lignin content. DNS analysis was performed to analyze the reducing sugar before and after the hydrolysis process measured using a spectrophotometer at a wavelength of 540 nm [10]. The content of reducing sugar from the best variable of hydrolysis process was tested by HPLC type Agilent 1100 Series equipped with Agilent 1260 Refractive Index Detector, positive polarity at 35 °C and column of Agilent Zorbax Carbohydrate 4.6 x 150 mm, 5 μm at 45 °C.

The XRD method was used to find the value of Crystallinity Index of lignocellulosic material. XRD will read the Crystallinity Index value of cellulose crystals in the solid sample. The intensity of amorphous cellulose can be seen at a 2θ angle of approximately 18 ° and crystalline cellulose at 22-24 °. The value of Crystallinity Index (CrI) was calculated using the Eq. (1):

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

Yield of reducing sugar was determined using the Eq. (2):

$$Reducing\ Sugar\ Yield = \frac{Reducing\ sugar\ conc. \times total\ volume\ of\ hydrolysate}{(\%cellulose + \%hemicellulose) \times weight\ of\ susbtrate} \quad (2)$$

3. Results and discussion

3.1. The effect of mixed culture pretreatment on lignocellulosic content of CPW

Pretreatment of the CPW was pretreated by mixed culture and single culture as the control. The underlying reason of the selection of such microorganisms was because of brown-rot fungi types such as *Aspergillus niger* usually can degrade cellulose and soft-rot fungi such as *Trichoderma reesei* can degrade cellulose and lignin [11]. Besides, it is also a fungi with the most cellulose degradation activity when compared to other cellulosic fungi [5]. Pretreatment was needed because it could damage the lignin structure so then cellulose can be easily degraded during hydrolysis processs [12].

Table 1. The cellulose content before and after pretreatment using AN:TR and BS:TR

Variable	*Lignin (%)*	Pectin (%)	*Cellulose (%)*	Hemicellulose (%)
BS:TR	1.96	0.98	65.82	24.7
AN:TR	2.05	1.04	67.05	26.18
Initial coffee pulp	6.82	2.18	58.62	21.96

Table 1 shows that there is a decrease of lignin content on pretreated sample of using mixed culture of BS:TR and AN:TR where lignin content of initial coffee pulp is 6.82 % and after pretreatment becomes 1.96 % and 2.05 % for BS:TR and AN:TR, respectively. The pectin content also decreased after the pretreatment process, where the initial pectin content was 2.18 % and after pretreatment became 1.04 % for AN:TR and 0.98 % for BS:TR. The result was also followed by the increase of cellulose and hemicellulose content. After pretreatment, it increased become 67.05 % for AN:TR and 65.82 % for BS:TR. Hemicellulose content after pretreatment increased to 26.18 % and 24.5 % for AN:TR and BS:TR, respectively. Decreasing levels of lignin and increasing of cellulose and hemicellulose levels indicate that the microbial used in this process have damaged the lignin layer and made cellulose and hemicellulose content increasing [12]. From the Table 1, the decreasing of lignin content in pretreatment by AN:TR is higher than the BS:TR. This result was in accordance with previous research conducted by [5] which reported that *Aspergillus niger* effectively degrade lignin and pectin while *Trichoderma reesei* effectively degrade lignin, so this mixed culture has degraded more lignin. Cellulose consists of D-glucopyranose and is bonded with the β-1,4-glycosidic bond. There is a hydroxyl bond in the cellulose macromolecule comprising of several intra- and inter-molecules hydrogen bond resulting in various cellulosic crystalline structure [13]. Since the cellulosic structure is so complex, it was necessary to find out how the crystalline structure of the substrate used by X-Ray Diffraction analysis. The crystalline structure of cellulose is imperfect, most of the cellulose structures that are located in the outer layer (less-ordered) are called amorphous, while the remainder which is located in the inside layer of cellulse (ordered) is called crystalline. The amorphous in the crystalline structure is the easier part of the cellulose substrate that can be degraded by enzymes [13]

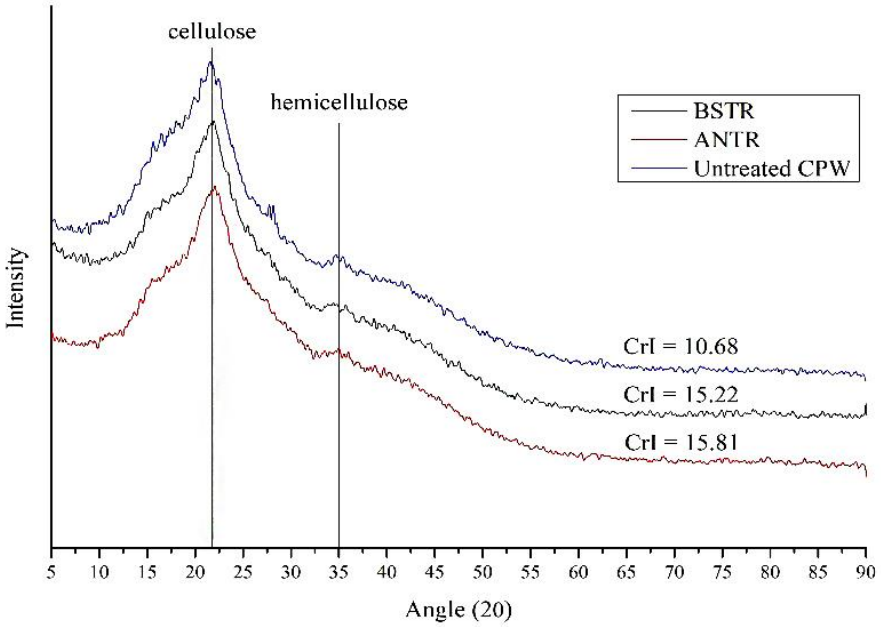

Figure 1. Graph of X-Ray Diffraction of CPW Before and After Pre-treatment using BS:TR and AN:TR

Figure 1 shows the results of XRD analysis for CPW before and after pretreatment. Sample peak sample before pre-treatment and after pre-treatment are shown in Figure 1 where the peak diffraction sample of AN:TR (1:1) at 22.045° and BS:TR (2:1) at 22.033°. Previous research stated that the diffraction peak at 2θ = 22° was the characteristic of cellulose crystalline I. After biological pretreatment, X-ray diffraction analysis with diffraction plane (021) and (002) has increased by the intensity. The result shown a general characteristic of the amorphous form of cellulose which is often found after pretreatment by dissolution and regeneration, where the area of the reflector plane (021) and (002) has increased [14]. From the Figure 1, at unpretreated CPW does not show any diffraction peak at 2θ = 35^o while the pretreatment sample does show the diffraction peak of 2θ = 35.07^o. In the hemicellulosic structure, the diffraction peak at 2θ = 35^o[15]. This result indicates that the hemicellulose content of the pre-treatment sample has increased. The increase of CrI value showed a loss of amorphous compound from the sample, the cellulose structure has been opened.Coffee pulp waste was obtained from Bangelan, East Java Indonesia. It was dried under the sunlight for approximately 7 days. Then the CPW was mashed using a grinding machine and sieved to get 120 meshes. The smaller substrate particles help the hydrolysis process to make the contact surface between the substrate and the enzymes larger and the lignocellulose content will be easier to be degraded. Before used, the CPW was dried using the oven at 105 ^{o}C to reduce moisture content.

3.2. The effect of enzyme and surfactant mixture towards enzymatic hydrolysis process

Enzymatic hydrolysis of coffee pulp aims to convert cellulose and hemicellulose into reducing sugars using a pure cellulase and xylanase enzyme. PEG 4000 was used to enhance the total reducing sugar after hydrolysis of lignocellulosic biomass [16], while Tween 80 was chosen due to the ability to influence on xylanase adsorption and desorption on/from lignin and occupies a portion of lignin hydrophobic surface [17]. The surfactant mass was designed based on research by [18] which states that non-ionic surfactants which added to the hydrolysis process will produce significant results, but if it is over than five grams, it will not have a significant effect due to the saturated solubility of the surfactant.

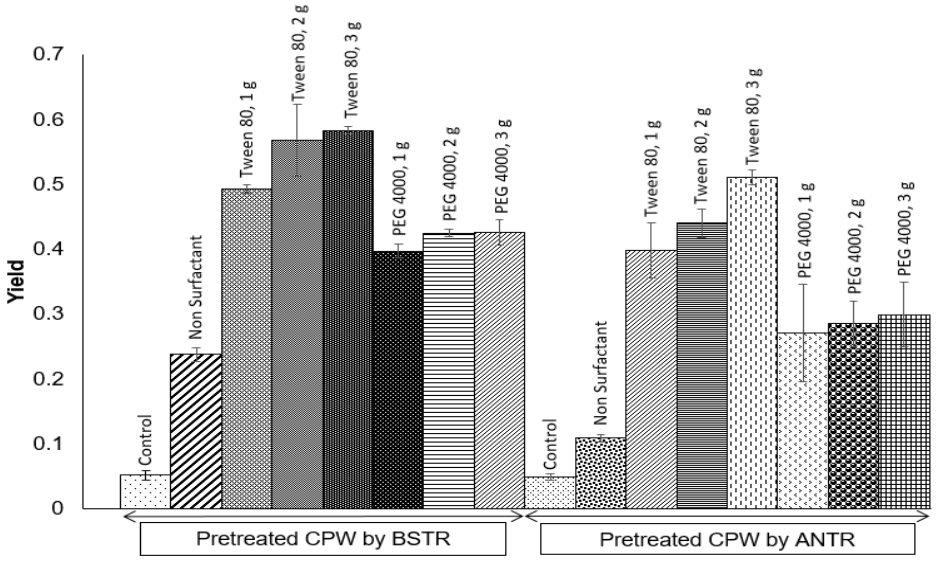

Figure 2. yield of reducing sugar from enzymatic hydrolysis

From the Figure 2, the higher mass of surfactants which has introduced into the sample would give the higher concentrations and yield of reducing sugars. This suggests that surfactants have a positive effect on enzymatic hydrolysis. Compared with non-hydrolysed samples, the other variables has significantly different of reducing sugar content in both with addition of surfactant and non-surfactant. The effects of surfactant addition have shown significant reducing sugar yield especially in the sample resulted from BS:TR pretretment with addition of Tween 80. This indicated that Tween 80 was a better surfactant than PEG 4000. Tween 80 shown the significant result than PEG 4000 because Tween 80 will increase the solubility of enzymes [19] and reduce the enzyme's absorption, so the enzyme can optimally work by encircling the substrate at first. Besides, addition of Tween 80 to the hydrolysis solutioncan increase the temperature by more than 10 °C due to the non-ionic bond formed. This condition will cause the enzyme to work more optimally, but this was not good for the culture that lived at optimum temperature of 35 °C [20].

In prereated CPW by BS:TR, the increase of reducing sugar yield in flask with non-surfactant and addition of 3 g of Tween 80 and 3 g of PEG 4000 was 52.535 %, 91,188 %, and 26.963 %. In the pretreated CPW by AN:TR, the increase of reducing sugar yield in flask with non-surfactant, addition 3 g of Tween 80, and 3 g of PEG 4000 was 49.499 %, 91.814 %, and 41.508 %, respectively.

From the explanation, it could be stated that the mixture of pure enzyme and surfactant might hydrolyze cellulose and hemicellulose in the pretreated CPW to be reducing sugar. The combination of a pure cellulase and pure xylanase enzyme could maximize the process of hydrolysis of the pretreated CPW, where the cellulose was hydrolyzed into glucose-reducing sugar by pure cellulase and hemicellulose was hydrolyzed by the xylanase into the xylose-reducing sugar.

3.3. The effect of microorganisms and surfactant mixture towards biologically hydrolysis process

The biologically hydrolysis has some benefits which are environmentally friendly, low energy requirement does not require chemicals and can avoid sugar degradation, resulting in higher yield [21]. In the present study, hydrolysis process was used *Aspergillus niger* and *Trichoderma viride* with mixture ratio of 1:1; 1:2 and 2:1. The use of *Aspergillus niger* and *Trichoderma viride* refers to previous studies which suggest that *Aspergillus niger* and *Trichoderma viride* are more potentially enzymatic cellulase than other microorganisms [22]. According to research by [23], *Aspergillus niger* is the most suitable microorganism for cultivation on the coffee pulp, where the microorganism can produce pectinase enzymes. *Aspergillus niger* is also known to produce cellulase enzyme with high specific activity [24]. In this study, the use of a mixture of *Aspergillus niger* and *Trichoderma viride* was based on these microorganisms can yield higher hydrolysis yields when compared to their single culture. The effective

addition of *Trichoderma viride* will produce the β-glucosidase enzyme that help *Aspergillus niger* to hydrolyze cellobiose which is an inhibitor of cellulase activity [11].
The biological hydrolysis will produce fungal cellulase using cellulose contained in the substrate as the carbon source [28]. Cellulosic microorganisms can build a synergistic relationship with non-cellulosic species in cellulosic waste, to make the synergistical interaction in cellulose degradation. Generally, endoglucanase and cellobiohydrolase will work synergistically to hydrolyze cellulose and biological hydrolysis will also produce microbial hemicellulase [24].

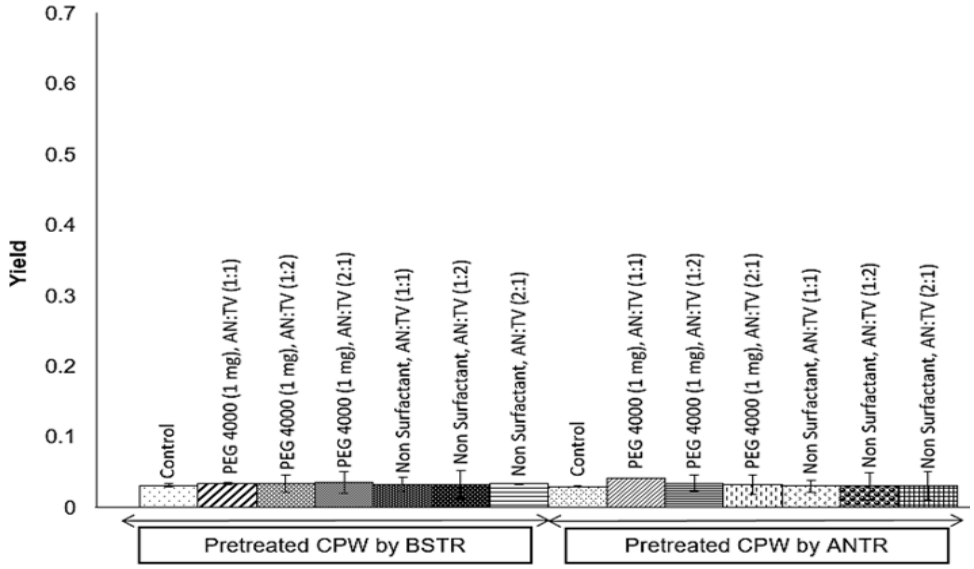

Figure 3. yield of reducing sugar from biological hydrolysis

Figure 3 shows the reducing sugar yield from biological hydrolysis. The yield has higher than the initial yield of pretreated CPW by BS:TR without hydrolysis which amounted to 0.031 (g/g) and 0.029 (g/g) for the pretreated CPW by AN:TR The hydrolysis by AN:TV in 2:1 ratio was resulted the highest reducing sugar yield from pretreated CPW by both of BS:TR (2:1) and AN:TR (1:1) which were 0.033 (g/g) and 0.031 (g/g), respectively. This was consistent with previous research that stated *Aspergillus niger* is the fungi with the highest specific activity to produce cellulase enzyme [24] since the amount of *Aspergillus niger* was higher in the culture, it would result in higher yield of cellulose. The obtained reducing sugar yields from pretreated CPW by AN:TR and hydrolyzed by AN:TV with ratio of 1:1, 1:2, and 2:1 without surfactant addition were 0.0302, 0.03 and 0.0306 (g/g), respectively. While, the reducing sugar yields with addition of surfactants shown a succession of 0.042, 0.034 and 0.032 (g/g) or increase to 39.07 %, 13.33 %, and 4.57 %, respectively. In the pretreated CPW by BS:TR and hydrolyzed by AN:TV with ratio of 1:1, 1:2, and 2:1 without surfactant were 0.032, 0.032 and 0.033 (g/g), respectively. When they were used the surfactant, reducing sugar yield has increased consecutively to 0.034, 0.034 and 0.035 (g/g) or increased 6.25 %, 6.25 %, and 6.06 %. These results were consistent with previous studies which suggest that the use of surfactants may increase delignification and increase the yield by fungal enzyme hydrolysis [25]. The mechanisms of surfactants that can explain their effect on enzymes are: (1) changing the substrate structure becomes more easily degraded by enzymes, (2) stabilizing the enzyme to avoid denaturation [26]. (3) increase the positive interaction between substrate and enzyme [20] and (4) decrease the number of unsupervised enzymes binding to lignin and other molecules involved in cellulase enzyme activity [27].

4. Conclusion

Production of reducing sugar from the coffee pulp waste using biological pretreatment process on a mixture culture between *Aspergillus niger*, *Bacillus subtilis*, and *Trichoderma reesei* followed by hydrolysis process using a mixture of pure cellulase and xylanase enzyme and using verious mixture

ratio of *Aspergillus niger* and *Trichoderma viride* was succesfully conducted. The optimum condition was obtained in biological pre-treatment process which the decrease of lignin and pectin content using BS:TR was higher than AN:TR In hydrolysis process, the optimum condition was obtained from pretreated CPW by BSTR with enzymatic hydrolysis and addition of Tween 80 of 3 g which produced yield of reducing sugar was 0.5831 %. Addition of surfactants have shown the significant reducing sugar yield, in case Tween 80 was better than PEG 4000. The increase of reducing sugar yield by addition of 3 g of Tween 80 could reach 91,188 % and 91.814 % in the pretreated CPW by BS:TR and AN:TR, respectively.

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