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Abstract. Bagasse is a solid waste from the sugar cane milling process in the sugar industry. Bagasse contains lignin, cellulose and hemicellulose, which through the fermentation process by *Phanerochaete chrysosporium* can produce crude cellulase, furthermore cellulase and other enzymes can be used in bagasse fermentation to produce bioethanol. The purpose of this study was to determine the effect of fermentation time and the addition of crude cellulase to the yield and concentration of bioethanol produced in bagasse fermentation. The research was carried out with the stages of the process: Preparation of raw materials (bagasse), Preparation of crude cellulase, Fermentation process, Product analysis (Bioethanol). The variables used in the experiment were fermentation time (96, 120, and 144 hours) and the addition of crude cellulase (10%, 20%, 30%, 40%, and 50% (v/v)). Analysis of crude cellulase activity using the DNS method, while the analysis of bioethanol concentration using the chromatography methods. The results showed that the maximum yield was 16.24% and the highest bioethanol concentration of 11.04% was obtained at the time of fermentation of 144 hours and the addition of crude cellulase by 50% (v/v).

1. Introduction

Bagasse is a solid waste from the sugar cane milling process in the sugar industry. Bagasse contains lignin, cellulose and hemicellulose, which through the fermentation process by *Phanerochaete chrysosporium* can produce crude cellulase, furthermore cellulase and other enzymes can be used in bagasse fermentation to produce bioethanol [1–3]. Bagasse (sugarcane bagasse) is included as lignocellulosic waste which can be used as raw material for making biofuels, such as bioethanol. Bagasse is biomass from vegetable. Biomass from vegetable source is renewable and has a great potential for energy generation. The composition of bagasse are cellulose 44.43 %, hemicellulose 22.90 %, and lignin 17.52 % [4]. On the other hand, *Phanerochaete chrysosporium* has the ability to produce cellulase enzymes from substrates containing cellulose and simultaneously produce enzymes that can break down lignin [5,6]. So there is no need for a delignification process to break down the lignin in bagasse [7].

In a study conducted by Rulianah et al [5] which used bagasse as raw material for crude cellulase production by *Phanerochaete chrysosporium* with fermentation times: 9, 11, 13, 15 and 17 days, and the concentration of bagasse added: 5, 6, and 7%. In this study, the highest crude cellulase activity was obtained at the longest fermentation time of 17 days. From the results of this study, further research was carried out for crude cellulase production by extending the fermentation time to 29 days, and the results

of crude cellulases were used for bioethanol production using the Simultaneous Sacharification and Fermentation (SSF) process [3]. In this study, the highest fermented bioethanol content was 9.22% [3]. These results are quite good when compared to using crude cellulases from other types of microbes, such as research conducted by Hernawan et al [4], which obtained ethanol levels of 6.83 ± 0.07%, and research [8], using *Pachysolen tannophilus* MTCC 1077, which produced ethanol with a concentration of 9.15 g/L at an incubation time of 72 hours.

The purpose of this study was to determine the effect of fermentation time and addition of crude cellulase produced by *Phanerochaete chrysosporium* on bioethanol levels produced from the fermentation process by Simultaneous Sacharification and Fermentation (SSF) using *Saccharomyces cerevisiae*. This research is a development from previous research [3], by adding CMC to the production medium of crude cellulase from bagasse using *Phanerochaete chrysosporium*, with the hope of increasing the activity of the crude cellulase enzyme produced.

2. Methods and materials

2.1. Materials and equipment

2.1.1. The materials used in this research. Sugarcane baggase was obtained from a local of sugar factory Kebun Agung, Malang, East Java, Indonesia. Potato Dextrose Agar (PDA) Difco ™ , $CaCl_2$, $MgSO_4$. H_2O, CTAB (Cetyltrimetyl Ammonium bromide), glucose, acetic acid, sodium acetate, CMC (Carboxy Methyl Cellulose), and ethanol p.a were purchased from Merck. H_2SO_4 98% was purchased from Smart Lab, ethanol 70%, cotton fatty, pH universal, NPK, spirit, distilled water, and vitamin B1 were purchased from local market. dry *Saccharomyces cerevisiae* (fermipan), *Phanerochaete chrysosporium* was cultured in Bioprocess Laboratory Politeknik Negeri Malang, Nitrogen Limited Media (NLM) consisting of: NPK (nitrogen, phosphorus, potassium) as a fertilizer, $MgSO_4 \cdot H_2O$, $CaCl_2$, glucose, and molasses from the Kebon Agung sugar factory, Malang, East Java.

2.1.2. The equipment used. Autoclave (Selecta), incubator shakers (New Brunswick Scientific CO., INC), incubator oven (Memmert), analytical balance (Yoke), vibrating screening (Retsch), spektofotometri UV-VIS (Perkin Elmer), furnace (Stuart), sentrifuge (Hitachi), water bath (Memmert), vacuum pump (Krisbow), oven (Memmert), microskop (Olympus), *Gas Chromatography* (GC) type HP 5890, measuring cup, erlenmeyer, measure pipette, pipette-sized (Schott Duran), micropipette (FinnpipetteTM F2), pH meter, RBF 250 mL dan 500 mL (Schott Duran), desiccator (unterdruck geprüft), and Whatman filter paper, and a set of distillation equipment (Preciso).

2.2. Pre-treatment of sugarcane baggase

Baggase was obtained from a local sugar factory Kebun Agung, Malang, East Java, Indonesia, dried up under sunlight, then reduced in size by a grinder to a size of ± 27-48 mesh, and the water level will be analysed.

2.3. Analysis water content of sugarcane bagasse

Weighing bagasse as much as 1 gram, then dry it in the oven for ± 1 hour at a temperature of 100-105^{o}C. After completion of the oven, the bagasse is cooled in a desiccator for ± 30 minutes. Then weighed. This treatment is repeated until a constant mass is obtained. Water content of sugarcane baggase is calculated by the following formula:

$$\text{Water content (\%)} = \frac{\text{initial weight (g)} - \text{final weight (g)}}{\text{initial weight (g)}} \times 100\% \quad (1)$$

Determination of levels of ADF, NDF, Hemicellulose, lignin and cellulose using the Van Soest method [7].

2.4. Production of crude cellulase

Crude cellulase production is carried out by making NLM consisting of NPK 6 g/L, $MgSO_4.7H_2O$ 0.5 g/L, vitamin B1 0.001 g/L, $CaCl_2$ 0.1 g/L, and glucose 10 g/L, dissolved in a pH buffer of 5 to 1 litter, then 330 mL of NLM are put into each 1 L erlenmeyer, add 7% (w/v) of bagasse, 0.5% (w/v) of CMC, and molasses of 0.4% (v/v). This media is sterilized at 121°C for 30 minutes. The sterilized media was cooled, then added inoculum *Phanerocahete chrysosporium* (10% (v/v) of the total volume). The inoculum contains 8.51 x 106 spores/mL. Then the incubation was carried out for 30 days, at a temperature of 35 °C and a shaker incubator speed of 150 rpm.

After the incubation process is complete, the results are filtered in a vacuum, separated between the supernatant and the biomass. Then the supernatant is analysed by the DNS method to determine the activity of crude cellulose enzymes.

2.5. Bioethanol production using Simultaneous Saccharification and Fermentation (SSF) Process

Ethanol production from bagasse media was carried out by making a Nutrient Medium consisting of 10 g/L $(NH_4)\ 2PO_4$, 0.5 g/L $MgSO_4.7H_2O$, 10 g/L glucose and 10 g/L yeast extract. Then dissolved with aquadest up to 1 L. The production of bioethanol is done using a brown bottle. In each bottle, 8% bagasse of the media volume and 100 ml Nutrient Medium were added, then sterilized at 121 °C for 30 minutes in autoclave. After being sterilized, crude cellulase was added to the media according to the specified variables, namely 10%, 20%, 30%, 40%, and 50% (v/v). Then, added dry *Saccharomyces cerevsiae* (fermipan) directly as much as 2% of the media volume (100 mL). After that, it was incubated (fermented) according to the specified variables, namely 96, 120 and 144 hours with a temperature of 30 °C. After the fermentation process, the fermented products were distilled at 100 °C to separate the liquid and solids, then the distillate results were measured, their mass and volume, and the ethanol content were analysed using Gas Chromatography (GC) type HP 5890.

3. Results and discussions

3.1. The results of the initial and final analysis of the bagasse, before and after fermentation

The results of the initial analysis of bagasse content using the Van Soest method [7] are shown in Table 1. In the table it is known that bagasse used as raw material has hemiseulose, cellulose and lignin levels that are different from previous researchers [9]. This difference occurs due to the levels of bagasse from different areas, different moisture content and different analysis methods, causing the results of different levels of lignin, cellulose and hemicellulose.

Table 1. Chemical compositions of the sugarcane bagasses before and after fermentation.

Component	Composition of bagasse before fermentation (%)	Composition of bagasse after fermentation (%)
Water	10,3	10,22
ADF	48,23	17,46
NDF	77,84	5,94
Hemicellulose	29,56	7,79
Cellulose	32,03	1,51
Lignin	15,67	0,39
Insoluble Ash	0,53	10,22

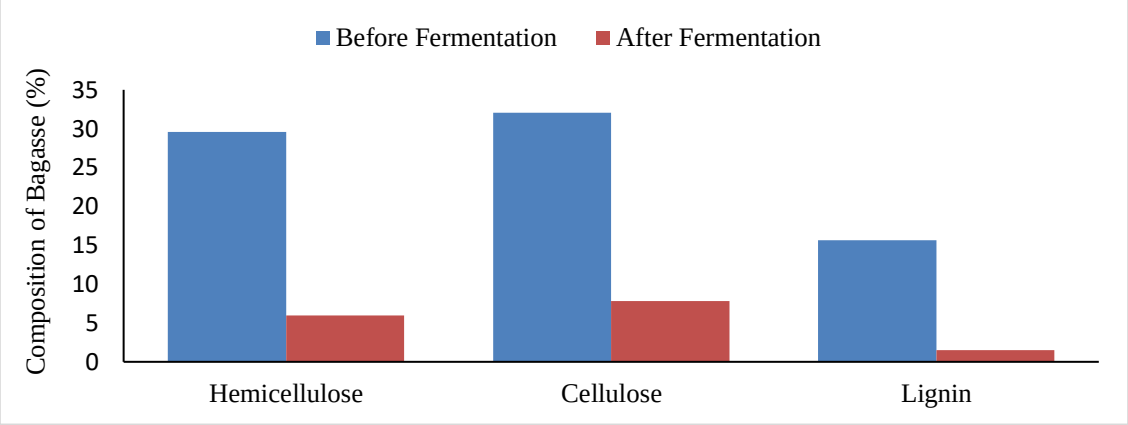

Figure 1. Hemicellulose, cellulose, and lignin contained in bagasse, before and after fermentation.

Based on Figure 1 above, there was a decrease in the levels of lignin, cellulose, and hemicellulose in bagasse after fermentation for 30 days. This was caused by the lignocellulose degradation process carried out by *Phanerochaete chrysosporium*. *P. chrysosporium* produces laccase enzymes, lignin peroxidase (LiP), and manganese peroxidase (MnP) which are used to degrade lignin. LiP activated by H_2O_2 can catalyze non-phenolic and phenolic lignin compounds. Meanwhile, MnP acts as a breaker of phenolic lignin units by oxidizing Mn^{2+} to Mn^{3+} [10,11] . Based on research conducted by Yakin et al [12] using the kakako fruit peel substrate, the enzyme activity of the *Phanerocahaete chrysosporium* fungus LiP and MnP was 0.22 U/mL and 0.09 U/mL, the activity of the enzymes LiP and MnP of *Pleurotus ostreatus* fungi. amounted to 0.13 U/mL and 0.05 U/mL, while the enzyme activity of the *Schizophyllum commune* mold LiP and MnP was 0.16 U/mL and 0.06 U/mL with fermentation time of 7 days. *Phanerochaete chrysosporium* also produces endoglucanase, exogluconase, and β-glycosidase enzymes [13–15]. These enzymes play a role in degrading cellulose into glucose. The endogilanase enzyme randomly attacks the β- (1,4) glycosidic bonds from the amorphous region and randomly cuts the internal bonds of the glycosidic chain. The exoglucanase enzyme plays a role in removing cellobiose from the ends of the free chain. The β-glycosidase enzyme acts to increase the hydrolysis of cellulose by converting cellobiose to glucose [16]. Meanwhile, hemicellulose has a complex oligomeric structure and branching pattern, making it difficult to degrade [17]. *Phanerochaete chrysosporium* mold produces the enzyme xylanase which acts to break down xylan into oligosaccharides. This causes a decrease in hemicellulose levels after fermentation.

In this study, there was a decrease in the levels of the bagasse components used. Decreased levels of bagasse content, namely 78.81% ADF, 77.57% NDF, 79.91% hemicellulose, 75.65% cellulose, and 90.36% lignin. According to Rulianah et al [5] shows that the best fermentation conditions are obtained at the addition of 0.8% CMC for 21 days with a decrease in lignin levels by 69.22% and a decrease in cellulose levels by 75.26%, on the fermentation of patchouli leaves. While the research conducted by Boonyuen et al [18], using the microorganisms *Aspergillus flavus* and *Penecillium citrinum* to degrade bagasse, obtained a decrease in lignin by 9.21%, cellulose by 9.08%, and hemicellulose 21.03% with fermentation time for 90 days. When compared with other studies, the decrease in bagasse content in the study was greater. This shows that using *Phanerochaete chrysosporium* can reduce levels of lignin, cellulose and hemicellulose higher than other types of fungi, such as *Aspergillus flavus*, *Penecillium citrinum*, and *Trichoderma viride*.

3.2. The effect of fermentation time and addition of crude cellulase to concentration of bioethanol in bagasse fermentation

The bioethanol fermentation process is carried out using *Saccaromyces cereviceae*. *Saccaromyces cereviceae* is a type of yeast in yeast that can convert sugar into other products in the form of alcohol (ethanol). The yeast produces invertase and zimase enzymes. Invertase enzyme functions to break down polysaccharides and sucrose which have not undergone the hydrolysis process into glucose (monosaccharide). While the zimase enzyme functions to convert glucose (monosaccharide) into alcohol products (ethanol) [19]. The following is the reaction for the formation of ethanol:

$$C_6H_{12}O_6 \xrightarrow{\textit{S. cereviceae}} 2\,CH_3CH_2OH + 2CO_2 \quad (2)$$

Glucose Ethanol

In this study, ethanol was prepared using the SSF method. The SSF method, which is a process in which cellulose hydrolysis and sugar fermentation are carried out simultaneously or simultaneously in one fermenter vessel [3,4,20–22]. In this study, the SSF method was carried out at 30 ^{o}C, 8% (w/v) substrate concentration, 10-50% (v/v) crude cellulase concentration and 2% (w/v) *Saccharomyces cereviceae* concentration. The fermentation process was carried out under anaerobic conditions for 96, 120 and 144 hours.

The results of bagasse fermentation to produce bioethanol using the SSF method, using crude cellulase produced by *Phanerochaete chrysosporium*, and yeast type *Saccaromyces cereviceae* are shown in Figure 2 and 3. This figure shows that the longer the fermentation time is up to 144 hours, the higher the bioethanol level produced, and it will increase the yield of bioethanol against bagasse. The figure also shows that the greater the amount of crude cellulase added up to 50%, it will increase the bioethanol content produced and will increase the yield of bioethanol against bagasse. Because the more amount of crude added will speed up the hydrolysis process, so that more sugar is available to be converted into bioethanol. Cellulase enzymes that hydrolyse cellulose have increased the amount of glucose so that *Saccharomyces cereviceae* will ferment glucose in a larger amount and produce higher levels of bioethanol as a result of its fermentation. However, at certain concentrations the rate of cellulose hydrolysis by enzymes will reach an optimal point so that the enzyme can no longer hydrolyse cellulose beyond its optimal point.

The fermentation time has a direct effect on the ethanol content produced. In this fermentation, fermentation time is closely related to the anaerobic growth of *Saccaromyces cereviceae*. In anaerobic conditions, *Saccharomyces cereviceae* tends to produce metabolite products in the form of enzymes that can catalyze glucose into bioethanol. It can be seen in Figure 2 that the ethanol content from 96 hours to 144 hours fermentation has increased, this is because up to 144 hours, *Saccharomyces cereviceae* is still alive and still produces enzymes that function as biocatalysts in the process of bioethanol formation. Based on the results of the research, the highest ethanol content was obtained at the addition of 50% crude and a fermentation time of 144 hours, which was 11.04% with a yield of 16.24%. According to research conducted by Silva [23], it shows that using a strain of *Kluyveromyces marxianus* as much as 30 FPU and a fermentation time of 72 hours using the SSF method from bagasse yields the highest ethanol concentration of 2.92%.

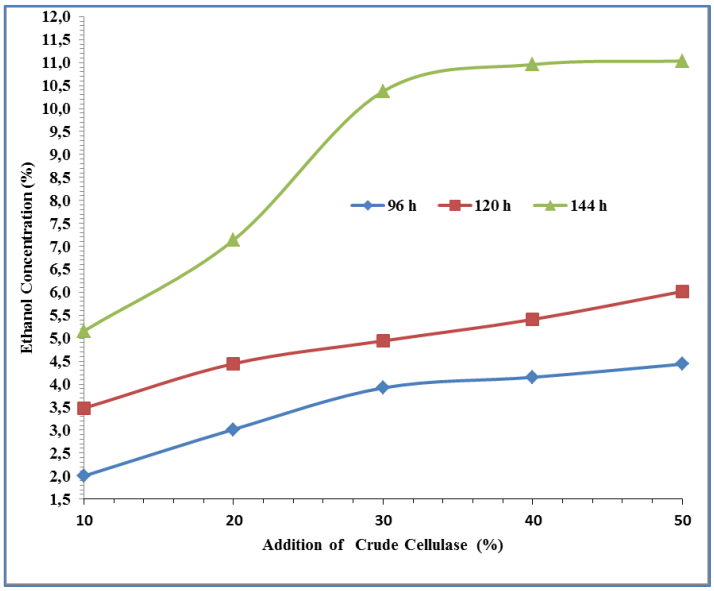

Figure 2. The relationship between the addition of crude cellulase and fermentation time to the ethanol content produced in the SSF method.

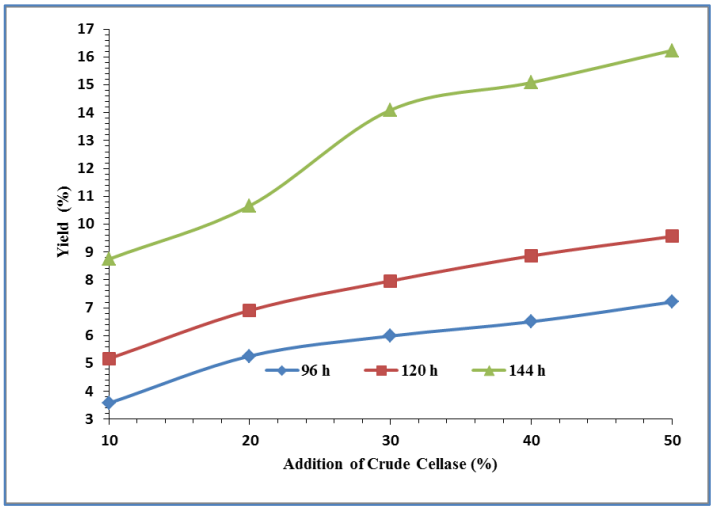

Figure 3. The relationship between the addition of crude cellulase and fermentation time to the yield ethanol.

Meanwhile, research conducted by Oktaviani [24], showed that the addition of 50 FPU commercial cellulase enzyme (Meicellase) and a fermentation time of 72 hours using the SSF method from alkaline delignified bagasse resulted in the highest ethanol concentration of 2.264%. Hashmi et al [25] obtained ethanol levels of 42.24 g/L equivalent to 4.224%. Hernawan et al [4] used the enzyme cellulase and combination of cellulase enzymes and β-glucosidase in the SSF process, resulting in bioethanol levels 5.87 ± 0.78% and 6.83 ± 0.07% respectively. If the results of this study are compared to the four previous studies above, it can be seen that the ethanol content in this study is much higher. This is due to the longer fermentation time and the cellulase enzyme used from the *Phanerochaete chrysosoporium* fungus, which is able to hydrolyse lignocellulose to glucose, which is higher than other fungi.

Meanwhile, bioethanol yield is directly proportional to bioethanol content, the higher the bioethanol content, the higher the yield produced. In this study, obtained higher results than previous studies [3], because in this study the media for crude cellulase production was added with CMC.

4. Conclusions

The results showed, the longer the fermentation time was up to 144 hours, and the higher the addition of crude cellulase from Phanerochaete chrysosporium to 50%, the higher the levels and yield of bioethanol produced. The best operating conditions were obtained when the addition of crude cellulase was 50% and the fermentation time was 144 hours, the ethanol content was 11.04% and the ethanol yield was 16.24%.

Suggestions for further research are to develop crude cellulase production technology that can increase the concentration of crude cellulase (crude cellulase activity) and develop bioethanol production from biomass (lignocellulose) raw materials.

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