Utilization of Bagasse for Bioethanol Raw Materials Using Crude Cellulase from *Phanerochaete Chrysosporium* with SSF Method

Sri Rulianah, Prayitno(✉), and Carita Ayu Maulidina

Department of Chemical Engineering, State Polytechnic of Malang, Malang, Indonesia
prayitno@polinema.ac.id

Abstract. Bagasse is one of the solid wastes from sugar factories which contains lignocellulose. Lignocellulose contains high levels of cellulose so that it has the potential as raw material to produce bioethanol. The crude cellulase produced by *Phanerochaete chrysosporium* has enzymes capable of degrading lignin, cellulose and hemicellulose simultaneously. The aim of the research is to analyses the effect of adding crude cellulose and *Saccharomyces cerevisiae* to the yield and concentration of bioethanol. The research was conducted by drying and reducing the size of the bagasse, cultivating of the *Phanerocheate chrysoporium*, fermenting the bagasse, and filtering. The resulting crude cellulase activity will be tested using the DNS method. The best crude cellulase activity is used to make bioethanol from bagasse as raw material. Simultaneous Saccharification and Fermentation (SSF) method is used in the production of bioethanol. The concentration of the bioethanol product was analysed using Gas Chromatography. The variables used are the addition of 6% bagasse, crude cellulose 40, 50, and 60% (v/v), *Saccharomyces cerevisiae* 1, 2, 3, 4, and 5% (w/v). In this study, the best conditions were obtained by adding 60% crude cellulase and 4% *Saccharomyces cerevisiae*, obtained bioethanol with a concentration of 9.18%, and a yield of 16.79%.

Keywords: Bagasse · Bioethanol · Crude cellulase · *Phanerochaete chrysosporium* · *Saccharomyces cerevisiae*

1 Introduction

Every year, the demand for fossil fuels is increasing. Fossil fuels are non-renewable natural resources because the formation process takes millions of years, while reserves in nature are depleted faster than the formation process. In addition, the excessive use of fossil fuels can also cause several negative impacts, such as increasing greenhouse gas emissions, which disrupt the environmental balance and increase fuel prices. This causes more and more people to look for alternative fuels to replace fossil fuels that are starting to run out [1].

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One of the alternative fuels that are being developed by the community today is bioethanol. Several materials can be processed into bioethanol, one of which is material containing lignocellulose. Bagasse is one of the agricultural wastes containing lignocellulose and its abundant supply. Bagasse contains 38.2% cellulose, 25% hemicellulose, and 24% lignin [2]; cellulose 54.8246%, lignin 22.3879%, hemicellulose 16.2387%, and water 11.3% [3].

Lignocellulose is a very promising raw material because it does not require additional land to produce raw materials and does not interfere with food and feed supplies. Ethanol production using bagasse as raw material requires low costs and can reduce pollution problems [4].

Cellulase enzymes can be used in the production of bioethanol because they can convert cellulose into glucose, where glucose is further converted into bioethanol. Cellulase production can use mold or bacteria. The molds that can produce cellulase are *Aspergillus niger* and *Trichoderma viride*, while *Bacillus*, *Cellulomonas*, and *Pseudomonas* are bacteria that can produce cellulase enzymes [5]. The ability of *Phanerochaete chrysosporium* is that it can produce enzymes that can degrade lignocellulose (lignin, cellulose, and hemicellulose) from substrates containing lignocellulose in one stage of the process so that it is more efficient [6]. Patchouli leaf fermentation using *Phanerochaete chrysosporium* for 21 days could reduce lignin levels by 69.22% [7] and was able to reduce cellulose levels by 75.26% [8]. This study showed that *Phanerochaete chrysosporium* was able to produce cellulase enzymes with high activity, in addition to producing enzymes that could degrade lignin and hemicellulose.

The incubation time and bagasse concentration affect the production of crude cellulose, where the incubation time of 17 days and the bagasse concentration of 7% (w/v) can produce the highest crude cellulase of 91.304 U/ml [7]. *Phanerochaete chrysosporium* can produce crude cellulase with greater activity than using other microbes. In a study on bioethanol production using bagasse with crude cellulase from *Phanerochaete chrysosporium*, using dry yeast, the best results were obtained with fermented ethanol with a concentration of 9.22% with a fermentation time of 96 h. The results of this study showed an increase in the concentration of ethanol when compared to previous studies, for example in the research of Bhatia and Johri, 2018, producing ethanol with a concentration of 9.15 g/L at 72 h incubation, with *Pachysolen tannophilus* MTCC 1077, and producing ethanol with a concentration of $6.83 \pm 0.07\%$ with commercial enzymes [9].

The bioethanol was made from sugarcane bagasse, with crude cellulase from *Phanerochaete chrysosporium* with sugarcane bagasse as a substrate. The variables that were changed were the amount of dry *Saccharomyces cerevisiae* and crude cellulase added by the Simultaneous Saccharification and Fermentation (SSF). The purpose of the research was to determine the effect of the concentration of *Saccharomyces cerevisiae* and crude cellulase added to the quality and yield of bioethanol. Meanwhile, the hypotheses used are H_o and H_1, where H_o states that there is an effect while H_1 states that there is no effect from the addition of *Saccharomyces cerevisiae* and cellulose crude.

2 Materials and methods

2.1 Materials Preparation

Bagasse produced from sugar factories around Malang City, East Java, Indonesia is dried using an Oven (type: Memmert) and then reduced in size using a crusher to form a powder. Bagasse powder is screened out at a size of ± 27 – 48 mesh and analysed for moisture, lignin, cellulose, and hemicellulose content.

The growth medium was made from NLM (glucose 10 g/L, CaCl2 0.1 g/L, vitamin B1 0.001 g/L, MgSO4.7H2O 0.5 g/L, and NPK 6 g/L) dissolved in a pH 5 solution buffer and added with bagasse powder 7% (w/v), molasses 0.25% (w/v), and CMC 0.5% (w/v). It is sterilized using an autoclave was carried out at 121 °C, and 1 bar for 30 min. The sterilized media then cooled to a temperature of ± 37 C, added *Phanerochaete chrysosporium* inoculum 10% (v/v)), and incubated in a shaker at a speed of 150 rpm, 37 °C, for 30 days. The incubation results are filtered using a vacuum pump, where the resulting filtrate is referred to as crude cellulase.

2.2 Production of Bioethanol with SSF Method

Bagasse powder at 6% w/v was added to the media consisted of 10 g/L glucose, 0.5 g/L $MgSO_4.7H_2O$, 10 g/L yeast extract and 10 g/L $(NH_4)_2PO_4$. The mixture of media and bagasse powder was sterilized in an autoclave at 121 C for 30 min. The sterilization results were cooled to 35 °C, then crude cellulase was added as much as 40%, 50%, and 60% (v/v), and *Saccharomyces cerevisiae* was added as much as (1, 2, 3, 4, and 5% (w/v)), and followed by a fermentation at a temperature of 30 °C for 6 days (144 h). The results of the fermentation were continued by a distillation to separate the impurities and the ethanol-water mixture which the distillation was carried out at a temperature of 100 °C. Then the distillate was analysed using gas chromatography (GC) type HP.5890 to determine the concentration of bioethanol.

2.3 Analysis Method

To analyse the water content in bagasse using the gravimetric method, analysis of bioethanol concentration using gas chromatography method, analysis of crude cellulase activity using the DNS method, analysis of lignocellulosic content used Van Soest Method.

3 Results and Discussions

3.1 Bagasse Composition

The composition of bagasse before and after the fermentation process using the Van Soest method is shown in Table 1. This difference occurs because the bagasse content of an area is different from the bagasse content of other regions due to differences in sugarcane quality, milling process quality, water content, and different analytical methods so that the results of the analysis of cellulose, hemicellulose, and lignin content from different

Table 1. Component of bagasse after 30 days of fermentation

Component	Initial fermentation (%)	After fermentation (%)
ADF	48,23	12,83
NDF	77,84	20,39
Hemicellulose	29,56	4,24
Cellulose	32,03	6,79
Lignin	15,67	4,24
Water	10,30	-

regions are not the same. To analyse the lignocellulosic content used Van Soest Methode which feed substances are classified into cell content and cell wall. Neutral Detergent Fibber (NDF) represents the cell content consisting of lignin, cellulose, hemicellulose, and proteins that bind to the cell wall while Acid Detergent Fibber (ADF) represents the cellulose and lignin of plant cell walls.

Table 1 shows that the components contained in lignocellulose decreased in concentration after the fermentation process by *Phanerochaete chrysosporium* for 30 days. This means that the enzymes produced by *Phanerochaete chrysosporium* can degrade the components in lignocellulose into crude cellulose. It can produce Manganese Peroxidase (MnP) and Lignin Peroxidase (LiP) enzymes, both of which are the main lignin-degrading enzymes [10].

The decrease in cellulose and hemicellulose content indicated that *Phanerochaete chrysosporium* produced cellulase and xylanase enzymes that could degrade cellulose and hemicellulose. *Phanerochaete chrysosporium* is a white rot fungus that produces lignocellulosic enzymes consisting of hydrolytic enzymes (a role in the degradation of cellulose and hemicellulose) and oxidative enzymes (a role in the degradation of lignin). In addition, the decrease in cellulose and hemicellulose indicates that *Phanerochaete chrysosporium* has succeeded in degrading lignin so that access to cellulose and hemicellulose reforms [11]. In this study, the presence of the cellulase enzyme was proven by analysis using the CMC method with DNS reagents, while the presence of the xylanase enzyme was only proven by a decrease in the hemicellulose content. Hemicellulose degradation involves enzymes that are almost the same as cellulose degradation. Hemicellulose is degraded into sugar monomers and acetic acid where xylan is the main carbohydrate found in hemicellulose. Xylanase is the main hemicellulose that hydrolysis the -1,4 bonds of the xylan chain. *Phanerochaete chrysosporium* mould produces Endo xylanase which plays a role in the breakdown of xylan into oligosaccharides [1].

3.2 Effect of the Amount of Crude Cellulase and Added Saccharomyces Cerevisiae on the Concentration and Yield of Bioethanol Produced

Bioethanol production in this study uses crude cellulase from bagasse as raw material using *Phanerochaete chrysosporium.* The resulting crude cellulase has an activity of

207.04 U/ml. In this bioethanol production process, dry *Saccharomyces cerevisiae* was used, the fermentation time was 144 h, and the substrate used was sugarcane bagasse. The operating conditions for the fermentation of bioethanol are using a temperature of 30 °C with a pH of 4.5, where this condition is the optimal condition for *Saccharomyces cerevisiae*. If the temperature used is too high, the yeast exponential phase will be shorter, the lower pH, the longer the required fermentation time, while the higher pH causes the ethanol concentration to decrease [12]. *Saccharomyces cerevisiae* is a type of yeast that is superior and can survive at various pH and temperature, so it is used in the production of bioethanol. In the fermentation process, factors that must be considered include temperature, pH, and glucose concentration because they greatly affect the production of ethanol and the specific growth rate of yeast [13].

In the production of bioethanol, yeast *Saccharomyces cerevisiae* is used which can convert sugar into ethanol due to the invertase and zymase enzymes, and crude cellulase to hydrolyse lignocellulose, which is produced by *Phanerochaete chrysosporium*. With the presence of these enzymes, *Saccharomyces cerevisiae* can convert sugars from both monosaccharide and disaccharide groups. If the sugar available in the substrate is a disaccharide sugar, the invertase enzyme will work to hydrolyse the disaccharide into monosaccharides. After that, the zymase enzyme will convert monosaccharides into alcohol and CO_2. The method used in the manufacture of ethanol is the Simultaneous Saccharification and Fermentation (SSF) method, in which *Saccharomyces cerevisiae* and crude cellulase are introduced into a process simultaneously which allows the glucose formed during enzymatic hydrolysis to be directly consumed by the yeast for conversion to bioethanol [14].

Figure 1 is the result of research which shows that the addition of crude cellulase and the addition of *Saccharomyces cerevisiae* influence the concentration of bioethanol produced. The greater the crude cellulase added, the more the concentration of bioethanol produced will increase. This shows that the hydrolysis of cellulose from sugarcane bagasse by crude cellulase produces glucose which is comparable to the glucose used by *Saccharomyces cerevisiae* to be converted into ethanol. The highest concentration of bioethanol was obtained at the highest crude cellulase addition variable, namely 60% and *Saccharomyces cerevisiae* by 4%, obtained bioethanol with a concentration of 9.18%, the lowest bioethanol concentration was obtained at 40% crude cellulase, and the addition of *Saccharomyces cerevisiae* was 1%, the bioethanol concentration was obtained at 1.30%. The higher the crude cellulase added, the higher the ethanol content produced, because the crude cellulase used is made from bagasse, so it contains enzymes that can degrade lignocellulose in bagasse and produce simple sugars. [15] stated that the concentration of the cellulase enzyme affects the production of bioethanol using the same amount of substrate because the raw cellulase enzyme will accelerate the hydrolysis process so that more sugar is produced, and more sugar is available to be fermented into ethanol. The addition of too many enzymes is not effective because it will increase production costs. Reference [16] showed that the rougher the active site of the enzyme in contact with the substrate (cellulose) also increased so that more and more cellulose was hydrolysed into glucose, causing glucose products to also stick to the active part. The enzyme site so that the contact surface area of the enzyme with the cellulose substrate is reduced.

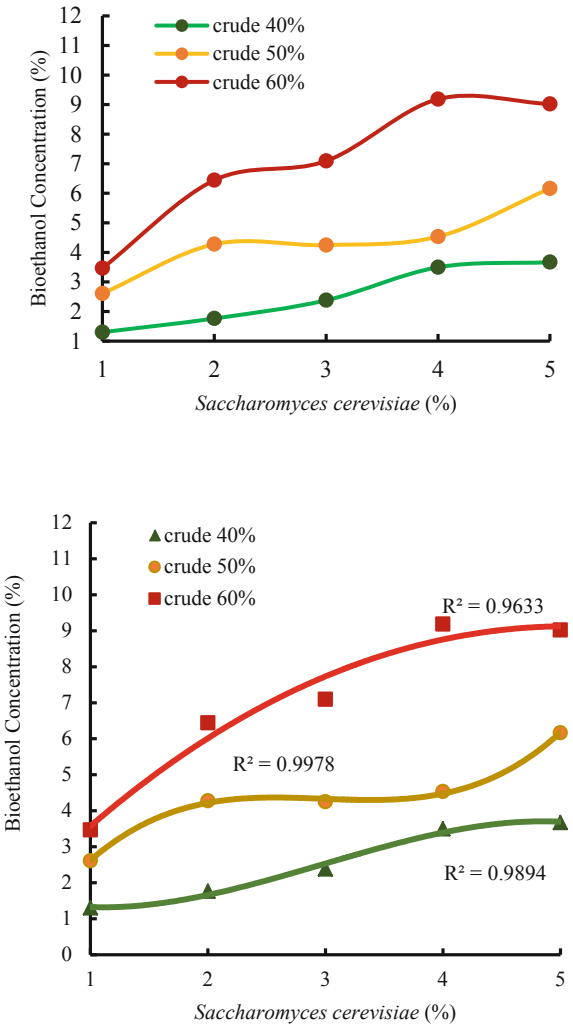

Fig. 1. The relationship between the addition of *Saccharomyces cerevisiae* (%w/v) and crude cellulase (%v/v) to the concentration of bioethanol.

Factors influencing bioethanol production include sugar concentration, pH, temperature, fermentation time, inoculum size, and agitation speed [17]. Temperature has a direct effect on the growth rate of microorganisms [18]. The temperature must be in accordance with the growth temperature of the microorganism, it should not be too high, and it should not be too low, because it will cause a stress factor for the microorganism [19]. Temperatures between 20 and 35 °C are the ideal temperature range for the fermentation process. The optimum temperature for free cells of *Saccharomyces cerevisiae* is ± 30 °C [10].

Figure 1 also shows that the higher the *Saccharomyces cerevisiae* added from 1 to 4%, the higher the bioethanol content produced. Because more and more *Saccharomyces cerevisiae* were added, the glucose converted into bioethanol also increased, but the addition of more than 4% would decrease the concentration of ethanol produced. In this study, the smaller the amount of *Saccharomyces cerevisiae* added, the smaller the concentration of bioethanol. This condition was obtained with the addition of *Saccharomyces cerevisiae* of 1% w/v, the smallest concentration of bioethanol was obtained, namely 1.30%. This condition indicates that if the amount of *Saccharomyces cerevisiae* added is insufficient to produce the zymase and invertase enzymes needed to catalyse the existing substrate, there will be an excess of substrate that is too high which will result in the reaction of ethanol formation being slower. Reference [20] stated that the amount of yeast (*Saccharomyces cerevisiae*) used in bioethanol fermentation affected the alcohol content produced. The higher the amount of yeast used, the higher the bioethanol content produced. In this research, the best condition for the addition of *Saccharomyces cerevisiae* was 4%, so more than 4% is not recommended. Reference [21] stated that in the manufacture of ethanol, the addition of yeast should not be too high, because it will cause a decrease in cell viability after the growth phase. Metabolic conditions and growth in cell populations that are too high are not expected because they cause disruption of access to nutrients, space limitations, and interactions between cells.

The highest ethanol concentration achieved in this study was 9.18%, where the bioethanol content produced was higher than previous studies because each researcher uses different types of enzymes for the hydrolysis process, also the addition of *Saccharomyces cerevisiae* is different, both in the phase and concentration of addition, also because other operating conditions are different [9, 15, 22].

The relationship between the addition of *Saccharomyces cerevisiae* and crude cellulase to the bioethanol yield is described in Fig. 2. The figure explains the relationship between the effect of adding crude cellulase of 40, 50, and 60%, and the addition of *Saccharomyces cerevisiae* from 1 to 5% on the resulting bioethanol yield.

The figure explains that the higher the addition of crude cellulase from 40% to 60% (v/v), and the greater the addition of *Saccharomyces cerevisiae* from 1 to 4% (w/v), the greater the % yield of ethanol produced. With the addition of *Saccharomyces cerevisiae* 5% (w/v), the yield showed a decrease. In this study, the lowest yield was 7.85%, with the addition of crude cellulase 40% (v/v), and the addition of *Saccharomyces cerevisiae* 1% (w/v). While the addition of 60% crude cellulase, and the addition of *Saccharomyces cerevisiae* by 4% (w/v), resulted in the highest yield of 16.79%. The ethanol content and ethanol yield are directly proportional, the higher the ethanol content produced, the higher the ethanol yield obtained.

The addition of *Saccharomyses cerevisiae* and crude cellulose to bagasse affects the concentration of bioethanol and yield, where the addition of *Saccharomyses cerevisiae* by 4% (w/v) and crude cellolase by 60% (v/v) produces bioethanol of 9.18% and yield of 16.79%.

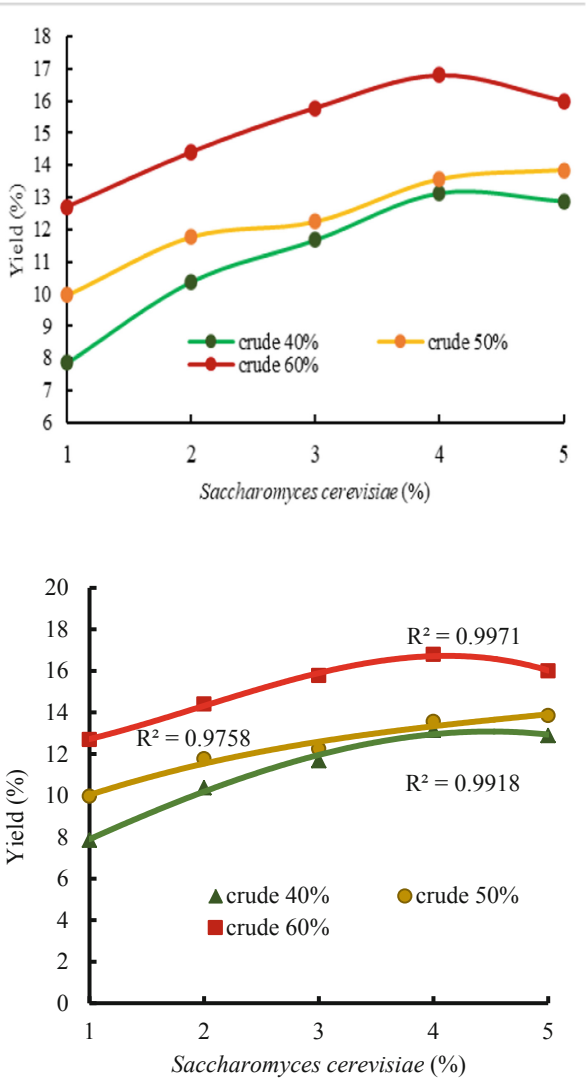

Fig. 2. Effect of addition of crude cellulase (%v/v), and *Saccharomyces cerevisiae* (%w/v) to the yield of ethanol.

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Authors' Contributions. SR conceived the original idea, SR and P screened and summarized all obtained literatures, evaluated the generation of tables and schemes, as well as analyzed the

bias of the study. The main text was written by SR and CR. The manuscript was initially written by SR, and the improved and revised by P. All authors read and approved the final manuscript.

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