Effect of *Saccharomyces cerevisiae* ATCC 9763 concentration and fermentation time on bioethanol content from corn stover crude cellulose substrate

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Abstract. Corn stover is a waste from the corn plant that dried in the fields after the corn cobs were harvested. From many corn wastes produced, there is very little utilization to corn stover. Delignified corn stover contains 65.46% cellulose, 14.58% hemicellulose, and 8.66% lignin. Lignocellulosic biomass is very difficult to biotransform, therefore it must be delignified to break the bonds between cellulose, hemicellulose, and lignin. Then the cellulose is converted into sugars by saccharification using crude cellulose enzymes so it can be converted into bioethanol through a fermentation process using simultaneous saccharification and fermentation (SSF) method. This study aims to determine the concentration of *Saccharomyces cerevisiae* ATCC 9763 and the optimum fermentation time in order to obtain high content of bioethanol from corn stover. Bioethanol production at different concentrations of *S. cerevisiae* and fermentation time uses a factorial randomized block design (RBD) consisting of two factors. The first factor was the concentration of *S. cerevisiae* which consisted of 3 levels, namely 3%, 5%, and 7% (v/v). The second factor was the fermentation time which consists of 4 levels, namely 24 hours, 48 hours, 72 hours, and 96 hours. Observed variables included pH value, total dissolved solids, reducing sugar content, and ethanol content. The data obtained were analyzed for its diversity using analysis of variants (ANOVA) and continued with the HSD Tukey. The results showed that the concentration of *S. cerevisiae* 7% (v/v) and a fermentation time of 96 hours was the best treatment to obtain a maximum ethanol content of 7.53 ± 0.330 g/L, with a final pH value of 4.25 ± 0.07, total dissolved solids 2.9 ± 0.14 °Bx and reducing sugar content of 0.334 ± 0.03 g/L. Increasing the concentration of *S. cerevisiae* and fermentation time can increase the ethanol content.

1. Introduction

Bioethanol is an alternative that is needed at present and in the future and will experience a significant increase in production due to the large number of raw materials that can be used for the production of bioethanol [1]. Bioethanol is an alcohol made by microbial fermentation [2], [3]. The raw material for bioethanol can come from biomass sources such as starch (corn, cassava, sorghum, etc.), sugar sources (molasses, sugar cane juice, coconut juice, and juice from various other plants) and cellulose sources (rice straw, corn straw, corn cobs, bagasse, etc.) [4], [5], [6].

According to the Badan Pusat Statistik (BPS) Indonesia corn production reached more than 19 million tons in 2019, so it is certain that it will produce a lot of corn waste. Due to the lack of public knowledge, at this point the use of corn waste in Indonesia is mostly limited just for animal feed, craft materials and briquette materials without the public being aware of the huge potential of corn waste, especially corn stover as raw material for making bioethanol in which there are several ingredients that
can be used in the production of bioethanol, namely lignocellulose (lignin, cellulose and hemicellulose) [7]. Lignocellulosic waste is very abundant, easy to obtain, cheap, inedible and has not been used optimally, giving it the potential to be used as raw material for renewable energy production in the future [1]. Delignified corn straw contains 65.46% cellulose, 14.58% hemicellulose, and 8.66% lignin [8]. Lignocellulosic biomass is very difficult to biotransform, both with microbes and enzymes [9] therefore it must be delignified to break the bonds between cellulose, hemicellulose and lignin. Then the cellulose is converted into sugars by saccharification [2], which after can be converted into bioethanol through a fermentation process. In industry, generally bioethanol can be fermented using \textit{Zymomonas mobilis} and \textit{Saccharomyces cerevisiae} [10]. Theoretically, \textit{Z. mobilis} has a higher conversion of sugar into ethanol compared to \textit{S. cerevisiae}, which is up to 97%, but \textit{S. cerevisiae} is more widely used as a biocatalyst in ethanol production because it can convert more variety of sugars and has a higher resistance to ethanol concentration [11].

In this study, the process of making bioethanol was carried out by going through several main process stages, namely delignification, simultaneous saccharification and fermentation (SSF) which is a combination of enzymatic hydrolysis and fermentation carried out simultaneously in one bioreactor and distillation. Cellulose saccharification in this study was carried out using crude cellulase enzymes produced using cellulolytic bacteria isolates B2S8 from previous studies.

In this study, evaluation was carried out on the concentration of \textit{S. cerevisiae} and the length of time of fermentation where time and inoculum concentration are two of the factors that affect fermentation in the production of bioethanol. The speed of ethanol fermentation is influenced by several factors such as the composition of the substrate, nutrients, concentration of inoculum, physiological state of the yeast, activity of enzymes, and the tolerance of the yeast to high sugar and alcohol concentration during fermentation [4]. The purpose of this study was to find the best combination between the concentration of \textit{S. cerevisiae} and the fermentation time to obtain maximum bioethanol content.

2. Materials and methods

2.1. Materials

The materials used in this study were corn stover type \textit{Zea mays saccharata} obtained from Sanur Denpasar, yeast \textit{Saccharomyces cerevisiae} ATCC 9763 from the Laboratory of Microbiology IPB University, crude cellulase enzyme from the Laboratory of Bioindustry and Environment, Faculty of Agricultural Technology Udayana University; citric acid, sodium citrate, sodium hydroxide, distilled water, 70% alcohol (Onemed), dextrose (Lihua Starch), peptone (Merck), yeast extract (Himedia), CMC (Merck), K$_2$HPO$_4$ (Merck), and CaCl$_2$ (Merck).

2.2. Preparation

The lignocellulosic waste of corn stover was cut into smaller sizes and then dried in an oven at 60°C for 48 hours, after that it was crushed using a blender, then the powder was sieved using a 60 mesh sieve [12], [8].

2.3. Delignification

Corn stover powder was soaked in a 4% (w/v) NaOH solution with a ratio of 1:10 (w/v) for 8 hours at room temperature, then the corn stover powder was washed using distilled water until it reached pH 7, then the powder was dried in an oven at 60°C temperature [8].

2.4. Inoculum preparation

Preparation of \textit{S. cerevisiae} inoculum was carried out using 250 mL and 1000 mL Erlenmeyer. YPD broth was made by mixing 6% dextrose, 0.5% peptone and 0.5% yeast extract into 100 mL distilled water. The media was sterilized at 121°C with a pressure of 1 atm for 15 minutes. Furthermore, 1 mL of \textit{S. cerevisiae} was put into the media, incubated at room temperature for 24 hours. Incubation was carried
out with a rotary shaker at a speed of 120 rpm, after which it was resuspended into 750 mL YPD broth and incubated with the same treatment as before.

Harvesting of \textit{S. cerevisiae} cells was carried out by centrifugation at a speed of 5000 rpm for 5 minutes at a temperature of 15°C. Then the \textit{S. cerevisiae} cells were washed using 0.85% NaCl solution and the cell concentration was adjusted to OD$_{660}$ 5 [13], [14], [6].

2.5. \textit{Simultaneous saccharification and fermentation} (SSF)

SSF was carried out using a bioreactor with a size of 250 mL. The fermentation medium was prepared by mixing 150 mL of 0.05M citrate buffer pH 4.8 with 15 g corn stover powder, 1 g/L K$_2$HPO$_4$, CaCl$_2$ 0.2 g/L and yeast extract 2.5 g/L. The media was sterilized at 121°C with a pressure of 1 atm for 15 minutes, then crude cellulase enzyme with CMCase and FPase activity was 0.082 IU/mL, 0.023 IU/mL respectively, added with a concentration of 20% and \textit{S. cerevisiae} ATCC 9763 inoculum with concentrations according to treatment (3%, 5%, 7% v/v). The SSF process was carried out with variations in time of 24, 48, 72, and 96 hours at a temperature of 35°C. after the SSF process is complete, it is filtered using a 250-micron filter [13], [15], [16]. Analysis of pH value, reducing sugar content [17] and total dissolved solids [18] was carried out at each time variation.

2.6. Distillation

Distillation was done by inserting the fermented solution into a distillation container and mounted on a reflux distillator. This process was carried out at 80°C to separate ethanol from the mixture [4]. In the end of this process, measurements of ethanol content were carried out using gas chromatography [19].

2.7. Data analysis

Observed variables included pH value, total dissolved solids, reducing sugar content, and ethanol content. The data obtained were analyzed for its diversity using analysis of variants (ANOVA) and continued with the HSD Tukey.

3. Result and Discussion

3.1. pH analysis

The results of variance showed that the treatment concentration of \textit{S. cerevisiae} and fermentation time had a very significant effect (p<0.01), while the interaction had no significant effect (p>0.05) on the pH value obtained during the fermentation process. The average value of pH in the fermentation process can be seen in Table 1.

| \textit{S. cerevisiae} Concentration (%) | Fermentation Time (Hour) | Average |
|----------------------------------------|--------------------------|---------|
|                                        | 24                      | 48      | 72      | 96      |
| 3                                      | 4.75 ± 0.071            | 4.55 ± 0.071 | 4.60 ± 0.000 | 4.45 ± 0.071 | 4.59 ± 0.125a |
| 5                                      | 4.60 ± 0.000            | 4.60 ± 0.000 | 4.50 ± 0.000 | 4.35 ± 0.071 | 4.51 ± 0.118b |
| 7                                      | 4.65 ± 0.071            | 4.45 ± 0.071 | 4.35 ± 0.071 | 4.25 ± 0.071 | 4.43 ± 0.171c |
| **Average**                            | **4.67 ± 0.076a**       | **4.53 ± 0.076b** | **4.48 ± 0.126b** | **4.35 ± 0.100c** |

Note: different letters behind the value in the same row or column indicate a very significant difference at the 5% error level (p≤0.05).

The results in Table 1 show a decrease in the pH value for each increase of \textit{S. cerevisiae} concentration. The highest average value of fermented pH was obtained from the treatment with a \textit{S. cerevisiae} concentration of 3% (v/v) which was 4.59 ± 0.125, followed by a concentration of 5% (v/v) 4.51 ± 0.118 and the lowest was obtained from concentration of 7% (v/v) that is 4.43 ± 0.171.
The fermentation time treatment showed a decrease in the pH value at each increase in the fermentation time. The highest average value of fermented pH was obtained from the 24 hours fermentation time treatment, which was 4.67 ± 0.076, followed by 48 hours at 4.53 ± 0.076 then 72 hours at 4.48 ± 0.126 and the lowest was obtained from the fermentation time of 96 hours at 4.35 ± 0.100.

Based on the data from Table 1, it can be concluded that a decrease in the pH value for every increase of \( S. \text{cerevisiae} \) concentration and fermentation time can occur because during the fermentation process it will produce acidic dissolved \( \text{CO}_2 \) gas (\( \text{H}_2\text{CO}_3 \)) [20]. The decrease in pH is also caused by fermentation producing organic acids, where during the fermentation process not only produces ethanol but also produces organic acid compounds such as lactic, acetic and pyruvic acids which are formed during fermentation so that it will have an impact on decreasing the pH value [21], [22].

3.2. Total dissolved solids analysis

The results of variance showed that the concentration of \( S. \text{cerevisiae} \) and the fermentation time between treatments had a significant effect (p<0.05) on the total dissolved solids value obtained in the fermentation process. The average value of total dissolved solids in the fermentation process can be seen in Table 2.

| \( S. \text{cerevisiae} \) Concentration (%) | Fermentation Time (Hour) |
|------------------------------------------|------------------------|
|                                          | 24                     |
|                                          | 48                     |
|                                          | 72                     |
|                                          | 96                     |
| 3                                       | 4.3 ± 0.141a           |
|                                          | 3.2 ± 0.283cd          |
|                                          | 2.7 ± 0.141de          |
|                                          | 2.6 ± 0.000e           |
| 5                                       | 4 ± 0.000ab            |
|                                          | 2.9 ± 0.141de          |
|                                          | 2.6 ± 0.000e           |
|                                          | 2.9 ± 0.141de          |
| 7                                       | 3.7 ± 0.141bc          |
|                                          | 2.9 ± 0.141de          |
|                                          | 2.7 ± 0.141de          |
|                                          | 2.9 ± 0.141de          |

Note: different letters behind the value in the same row or column indicate a very significant difference at the 5% error level (p≤0.05).

Total dissolved solids are the amount of cellulose that has been successfully converted into glucose and fermentation media that dissolved in water [23].

Based on the data obtained in Table 2, it shows that the total dissolved solids in the fermentation process have increased from 0 to 24 hours fermentation where the average value of total soluble solids for 0 hours fermentation for the treatment of \( S. \text{cerevisiae} \) concentrations 3%, 5%, 7% respectively 2.5°Bx, 2.4°Bx and 2.6°Bx. The decrease from 24 hours to 48 hours occurred because \( S. \text{cerevisiae} \) consumed glucose and fermentation media which was characterized by a decrease in total dissolved solids from each variation of \( S. \text{cerevisiae} \) concentration 3%, 5%, and 7% and tended to be constant at 48 to 96 hours.

Cellulase enzyme activity can be seen from the fermentation time 0–24 hours where there is an increase in total dissolved solids which indicates that the enzyme can saccharify cellulose into glucose. In the 0–24 hours period, \( S. \text{cerevisiae} \) was still in the adaptation phase and produced a small amount of ethanol where the ethanol produced is a growth-associated product [24]. The significant decrease of total dissolved solids during fermentation time of 24–48 hours is thought to be because during the fermentation process, the fermentation medium and also the saccharified sugar, which is the dominant component of soluble solids, are metabolized by \( S. \text{cerevisiae} \) into ethanol and \( \text{CO}_2 \) [20]. Total dissolved solids tended to be constant at 48–96 hours at which time sugar consumption by \( S. \text{cerevisiae} \) was already in the stationary phase, comparable to the sugar produced from the saccharification of crude cellulase enzymes so the fermentation by \( S. \text{cerevisiae} \) could continue and followed by the increase of ethanol content [25].

3.3. Reducing sugar analysis
The results of variance showed that the concentration of *S. cerevisiae* and fermentation time between treatments had a very significant effect (p<0.01) on the value of reducing sugar content obtained in the fermentation process. The average value of reducing sugar content in the fermentation process can be seen in Figure 1.

![Figure 1. The average reducing sugar value of the fermented corn stover on the treatment of *S. cerevisiae* concentration and fermentation time](image)

In the enzymatic saccharification of cellulose, cellulase degrades cellulose to produce glucose. The efficiency and effectiveness of the hydrolysis process can be measured through the level of reducing sugar production [17].

Based on the data obtained in Figure 1 shows that the value of reducing sugar content in the fermentation process has increased significantly at 0–24 hours of fermentation. The activity of the cellulase enzyme can be seen in that time span where there is a significant increase in reducing sugar levels which indicates that the enzyme can saccharify cellulose into glucose. Reducing sugar levels decreased significantly from 24–48 hours this occurred because *S. cerevisiae* consumed glucose and converted it into ethanol and tended to be constant at 48–96 hours because *S. cerevisiae* had entered the stationary phase where ethanol production reached its maximum at the time of microorganisms are in the stationary phase, this is also because the amount of sugar produced by the cellulase saccharification is equivalent to the amount of sugar consumed by *S. cerevisiae* followed by an increase on ethanol content. Previous research states that the value of reducing sugar saccharified from crude cellulase enzymes with CM-Case activity of 0.074 IU/mL and FP-ase 0.024 IU/mL on corn cobs, decreased during fermentation time of 24 to 48 hours and tended to be constant at 48 to 48 hours. One hundred and twenty hours where the decrease in reducing sugar content is equivalent to the increase on ethanol content produced [25]. The decrease in the value of reducing sugar content will be followed by an increase in the level of ethanol produced in the fermentation process [5].

Saccharification control using 20% crude cellulase enzyme without *S. cerevisiae* with a pH of 4.8 and a temperature of 35°C resulted in a reducing sugar concentration that continued to increase from 0, 24, 48, 72 to 96 hours of incubation with a value of 0.174 g/L, 0.674 g/L, 1.135 g/L, 1.193 g/L and 2.074 g/L. Based on these results, it can be concluded that crude cellulase enzyme can still saccharify cellulose into glucose up to 96 hours of incubation. The difference in glucose levels between the saccharified control and SSF treatment at 24 hours resulted in higher glucose levels of 1.490 g/L compared to the control of 0.674 g/L. SSF process can increase the glucose levels produced because of the inhibitory effect caused by the high levels of glucose attached to the active site of the enzyme, where *S. cerevisiae*
will actively consume glucose so the active site of the cellulase enzyme will always be ready to convert cellulose to glucose [26].

Generally, cellulase enzymes can work optimally in a pH range of 4.5–5.5. The decrease in pH that occurs during fermentation is suspected can result in decreased enzyme performance and inhibit the saccharification process of cellulose into glucose which can be seen from the decreased reducing sugar content during 96 hours of fermentation, this results in a small amount of ethanol produced.

3.4. Bioethanol Analysis

The results of variance showed that the concentration of *S. cerevisiae* had a significant effect (p<0.05) while the fermentation time had a very significant effect (p<0.01) and the interaction between treatments had no significant effect (p>0.05) on the ethanol content obtained. The average value of ethanol content in the fermentation process can be seen in Figure 2.

![Figure 2](image.png)

**Figure 2.** The average ethanol content of the fermented corn stover on the treatment of *S. cerevisiae* concentration and fermentation time

The results in Figure 2 show an increase in the ethanol content for each increase in the concentration of *S. cerevisiae*. The lowest average value of fermented ethanol content was obtained from the treatment with a concentration of *S. cerevisiae* 3% (v/v) which was 3.35 ± 2.005 g/L, followed by a concentration of 5% (v/v) which was 4.06 ± 2.278 g/L and the highest was obtained from a concentration of 7% (v/v) that is 4.56 ± 2.621 g/L.

The fermentation time treatment showed an increase in the ethanol content at each increase in the fermentation time. The lowest average value of fermented ethanol content was obtained from the 24 hours fermentation time treatment, namely 1.36 ± 0.297 g/L, followed by 48 hours at 2.94 ± 0.431 g/L then 72 hours at 5.07 ± 0.753 g/L and the highest was obtained from the fermentation time 96 hours by 6.58 ± 0.969 g/L.

The increase in ethanol content occurred every increase in the concentration of *S. cerevisiae* and the increase in fermentation time. During 24 hours of fermentation, the ethanol content produced was low, because *S. cerevisiae* was still in the adaptation phase so that it had not yet actively converted glucose into ethanol but was able to produce ethanol in the form of growth-associated products [24]. This can be seen from the total dissolved solids and reducing sugar which increased during 24 hours of
fermentation where the saccharified glucose got a significant increase because *S. cerevisiae* was still in the adaptation phase and had not converted much sugar. After 24 hours of fermentation, the total soluble solids and reducing sugar levels decreased because *S. cerevisiae* was already actively converting sugar to ethanol, it can be seen in Figure 2. The ethanol content produced continued to increase after 24 hours of fermentation.

Based on the data from Figure 2, it can be concluded that an increase in ethanol content for every increase in *S. cerevisiae* concentration and fermentation time can occur because the higher levels of *S. cerevisiae* given will affect the interaction of *S. cerevisiae* and nutrient sources, and the longer the fermentation time, *S. cerevisiae* will enter a phase where ethanol production will reach its peak, namely the stationary phase. However, if the fermentation is too long, the nutrients in the substrate will be depleted and *S. cerevisiae* can no longer ferment glucose. Each increase in the concentration of *S. cerevisiae* and fermentation time, the higher the ethanol content produced, because the higher concentration of *S. cerevisiae* given and fermentation time the more glucose resulting from saccharification can be converted into ethanol [27], [28].

Fermentation control with *S. cerevisiae* 7% (v/v) and 15% glucose for 96 hours resulted in an ethanol content of 59.57 g/L. This proved that during 96 hours of fermentation, *S. cerevisiae* was able to actively convert 22.5 g of glucose into 11.33 mL of ethanol. The low levels of ethanol produced can occur due to several factors, including the evaporation of the ethanol produced due to the length of storage before analysis, the low levels of reducing sugar produced due to low enzyme activity, decreasing of pH which affected the enzyme activity along the fermentation process and the amount of *S. cerevisiae* inoculated that affected many substrates are used for metabolism instead of fermenting glucose into ethanol.

4. Conclusion
Concentration of *S. cerevisiae* and fermentation time affect the pH value and ethanol content of corn stover crude cellulose substrate fermentation. The interaction between treatments affects the total dissolved solids and reduces sugar. The highest ethanol content was obtained by treatment with a 7% (v/v) concentration of *S. cerevisiae* and a fermentation time of 96 hours which was 7.53 ± 0.330 g/L, with a final pH value of 4.25 ± 0.071, total dissolved solids 2.9 ± 0.141°Bx and reducing sugar content of 0.334 ± 0.027 g/L. Based on the ethanol content produced, corn stover substrate has potential as a raw material for bioethanol production in the future.

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