Bioethanol production from sugarcane molasses by instant dry yeast (effect of pretreatment and fermentation temperature)

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Abstract. Some bioethanol industries in Indonesia that use sugarcane molasses as raw material often face low level of ethanol content from fermentation process. The molasses condition and the temperature fluctuation are supposed to be the cause. This research aimed to determine the effect of sugarcane molasses pre-treatment with H2SO4 and fermentation temperature in bioethanol production by using instant dry yeast of Saccharomyces cerevisiae. Factorial Randomized Block Design with 2 factors was used in the research. The first factor was pre-treatment effect in sugarcane molasses (with and without pre-treatment) and the second was fermentation temperature (29°C, 32°C and 35°C). The data were analysed using analysis of variance or ANOVA and continued with further test Duncan Multiple Range Test or HSD (Honestly Significant Difference) with confidence interval of 5%. The best treatment was determined using Multiple Attribute Test. Results showed that sugarcane molasses pre-treatment with H2SO4 and fermentation temperature had a significant effect (α= 0.05) on bioethanol production. The best result was obtained from sugarcane molasses medium that given H2SO4 pre-treatment and fermentation temperature of 32°C with total decrease value of total soluble solids (% brix) of 10.9 % brix, total decrease sugar of 12.15%, reducing sugar consumption of 57.21 g/L, ethanol content of 8.30 % and yield ethanol of 68.67 %.

1. Introduction
Sugarcane molasses is one of the agricultural byproducts whose presence quite abundant in Indonesia. The availability of sugarcane molasses is in line with sugarcane productivity, where in Indonesia has high sugarcane productivity with a total production reaching 2,715,883 tons in 2016 [1]. One of product that can be produced from sugarcane molasses is bioethanol. Bioethanol produced from the fermentation process with the help of microorganisms such as yeast and bacteria [2, 3].

Some bioethanol industry in Indonesia that use sugarcane molasses as raw material often face low level of ethanol content from fermentation process. This causes the distillation process to become less efficient. Sugarcane molasses condition and the temperature fluctuation are supposed to be the cause of low level ethanol content. Sugarcane molasses still contain high amounts of minerals, including calcium. Calcium inhibits the fermentation process because it acts as an inhibitor of the
invertase enzyme which breaks sucrose into glucose and fructose, where glucose is then used by yeast cells to be converted into ethanol [4]. In addition, calcium can inhibit the action of magnesium ions which is cofactors of nearly 300 enzymes in cell biochemical processes [5]. A high calcium concentration in sugarcane molasses indicating the need of pretreatment process.

Fermentation temperature is also one of the factors that influence the ethanol content. Temperature greatly affects the enzymatic activity and turgidity of the yeast cell membrane [6]. A high fermentation temperature can affect the fermentation process to become less efficient. The purpose of this study was to determine the effect of the pretreatment process and fermentation temperature on the production of bioethanol by using instant dry yeast of Saccharomyces cerevisiae.

2. Materials and Method
2.1. Materials
The main materials used in this research was sugarcane molasses from PT PG Rajawali II, West Java, Indonesia, sodium hydroxide, distilled water (Hydrobat), Anthrone (Merck), 3.5-dinitrosalilic acid (DNS) (Sigma), Sodium potassium tartaric (Sigma), Na$_2$SO$_3$ (Sigma), Sodium oxalate (Sigma), Pb Acetate, PDA (Merck), yeast extract, soy peptone, ammonium sulfate, tween 20, alcohol 70%, spirits.

2.2. Methods
Randomized Block Data Design with two factors was used in this research. The first factor was pretreatment effect in sugarcane molasses (with and without pretreatment) and the second was fermentation temperature (29°C, 32°C and 35°C). The data were analysed using analysis of variance or ANOVA and continued with further test Duncan Multiple Range Test or HSD (Honestly Significant Difference) with confidence interval of 5%. The best treatment was determined using Multiple Attribute Test. There were several steps of bioethanol production, start from making pretreated fermentation medium, making seed culture and bioethanol production.

2.2.1 Pretreatment of sugarcane molasses fermentation medium with sulfuric acid
Sugarcane molasses were weighed and dissolved in water with a ratio of 1:1 (w/v), then a concentrated 96% H$_2$SO$_4$ was added until the pH reaches 3.9. The solution was heated for 5-10 minutes, then precipitated for 24 hours. The precipitate was filtered and discarded. Filtrate was added to sodium hydroxide until returns to the initial pH. Filtrate was then diluted with water to sugar concentration of 25 %brix. Sugarcane molasses solution with sugar concentration 25 %brix was mixed with 0.1% (w/v) of yeast extract and then sterilized 121°C 15 minutes.

2.2.2 Seed culture solution
Sugarcane molasses solution with sugar concentration 2.5 % brix mixed with yeast extract 0.5% (w/v), soy peptone 0.5% (w/v) and then sterilized 121 °C 15 minutes. Sterilized sugarcane molasses solution then added with 1% (w/v) instant dry yeast and tween 20. The solution was aerated for 24 hours by using air pump.

2.2.3 Bioethanol production
A total of 25% (w/v) seed culture was added into the glass jar, then added 30% (w/v) sugarcane molasses solution with sugar concentration 25 % brix (Feeding I). The mixture of the solution then put into the incubator (temperature 29 °C, 32 °C and 35 °C) and fermented for 6 hours. After 6 hours, 30% (w/v) sugarcane molasses solution with sugar concentration 25 % brix (Feeding II) added to the glass jar, then fermented until 48 hours. The fermented liquid then distilled with a simple method distillation. Distilled fermented liquid then measured using a density meter. The ethanol content of fermentation results obtained from calculations using the formula $V_1 \times M_1 = V_2 \times M_2$. 

\[ V_1 \times M_1 = V_2 \times M_2 \]
3. Results and Discussion

3.1. Characteristic of Raw Materials
Parameters of analysis for sugarcane molasses include total soluble solids, total sugar, reducing sugar, ash and percentage of calcium. The result of sugarcane molasses characterisation analysis is presented in Table 1.

**Table 1. Characteristics of sugarcane molasses from PT PG Rajawali II**

| Parameter                      | Analysis Result |
|--------------------------------|-----------------|
| Total Soluble Solids (% brix)  | 80.1            |
| Total Sugar (%)                | 57.01 ± 0.47    |
| Reducing sugar (g/L)           | 20.48 ± 0.38    |
| Ash (%)                        | 7.46 ± 0.05     |
| Calcium (%)                    | 0.60 ± 0.01     |

Note: The number after ± is the standard deviation value

3.2. Total Soluble Solids (% brix)
During the fermentation process, a decrease in total soluble solids from fermented liquids was observed, with the values in the range of 9.10 - 10.80. ANOVA analysis results showed that H₂SO₄ pretreatment and fermentation temperature had a significant effect on the decrease of total soluble solids during the fermentation of sugarcane molasses. Table 2 shows a decrease of total soluble solids in various treatments.

**Table 2. Effect of pre-treatment and fermentation temperature on total soluble solids (% brix)**

| Treatment                  | Total Soluble Solids (% brix) |
|----------------------------|-------------------------------|
| 29 °C ± 1 °C               |                               |
| H₂SO₄ pre-treatment        | 10.80 ± 0.16                  |
| Without pre-treatment      | 10.50 ± 0.25                  |
| 32 °C ± 1°C                |                               |
| H₂SO₄ pre-treatment        | 10.90 ± 0.09                  |
| Without pre-treatment      | 9.70 ± 0.19                   |
| 35 °C ± 1°C                |                               |
| H₂SO₄ pre-treatment        | 9.50 ± 0.19                   |
| Without pre-treatment      | 9.10 ± 0.09                   |

Calcium inhibits the fermentation process because it acts as an inhibitor of the invertase enzyme which breaks sucrose into glucose and fructose, where glucose is then used by yeast cells to be converted into ethanol [4]. In addition, calcium can inhibit the action of magnesium ions which is cofactors of nearly 300 enzymes in cell biochemical process. Pre-treatment process will reduce calcium levels so the fermentation process becomes more optimal and causes a higher decrease in total soluble solids. Fermentation temperature is related to the activity of cell metabolism during the fermentation process. The optimal temperature for yeast cell growth was ranged between 25 - 30 °C and a maximum range between 35 – 47 °C. A good temperature for ethanol fermentation was in the range of 31 – 33 °C [7].

3.3. Total Sugar Value (%)
Decrease of total sugar are observed during fermentation process for 48 hours. The results of analysis of variance (ANNOVA) showed sugarcane molasses fermentation medium that given pretreatment H₂SO₄ and fermentation temperature had a significant effect on the decrease of total
sugar value during fermentation. Table 3 showed decrease of total sugar value during fermentation process on various treatment.

| Treatment                        | Total sugar (%) | 0 | 6FI* | 6FII** | 48 |
|----------------------------------|-----------------|---|------|--------|----|
| H2SO4 pre-treatment 29 °C ± 1°C | 16.18           | 14.01 | 17.93 | 8.22   |
| Without pre-treatment           | 15.94           | 13.30 | 17.53 | 6.87   |
| H2SO4 pre-treatment 32 °C ± 1°C | 16.18           | 13.26 | 17.71 | 8.48   |
| Without pre-treatment           | 15.75           | 12.07 | 17.20 | 7.17   |
| H2SO4 pre-treatment 35 °C ± 1°C | 15.75           | 13.93 | 19.21 | 7.67   |
| Without pre-treatment           | 16.18           | 12.51 | 18.48 | 8.06   |

Note: * in the end of feeding I process  
** after feeding II process

In the beginning of fermentation process, total sugar for all treatment range between 15—17%. Total sugar value can affect both the growth of yeasts and the production of ethanol. If the goal is the production of biomass, it is convenient that the process be conducted with low concentration of sugars and aeration, to avoid that, even in the presence of oxygen, respiration is repressed in high sugar concentrations (CRABTREE effect) (3). Total decrease of sugar used during the fermentation process ranges from 11-13%.

3.4. Reducing Sugar (g/L)
Consumption of reducing sugar during fermentation are observed for 48 hours. Reducing sugar consumption by yeast cell during fermentation range between 47.78—57.21 g/L. The results of analysis of variance (ANOVA) showed sugarcane molasses fermentation medium that given pretreatment H2SO4 and fermentation temperature had a significant effect on the total consumption of reducing sugar during fermentation. Table 4 shows total consumption of reducing sugar during fermentation in various treatments.

| Treatment                        | Consumption of reducing sugar (g/L) |
|----------------------------------|-------------------------------------|
| H2SO4 pre-treatment 29 °C ± 1°C | 53.61 ± 0.76                        |
| Without pre-treatment            | 48.98 ± 0.34                        |
| H2SO4 pre-treatment 32 °C ± 1°C | 57.21 ± 1.34                        |
| Without pre-treatment            | 53.33 ± 1.10                        |
| H2SO4 pre-treatment 35 °C ± 1°C | 49.35 ± 1.15                        |
| Without pre-treatment            | 47.78 ± 0.87                        |

Note: The number after ± is the standard deviation value

Reducing sugar contained in the fermentation medium is consumed by yeast cells during the fermentation process. Reducing sugars, especially glucose, are converted to ethanol through the metabolic pathway of EMP (Embden Mayerhoff Parnas) under anaerobic conditions. The EMP metabolic pathway will produce 2 ATP (16 kcal). The initial stage of the metabolic pathway is almost the same as glucose metabolism in aerobic conditions, where this pathway also passes through
the glycolysis pathway at an early stage [8]. The difference is that pyruvic acid produced from the glycolysis stage will be converted to ethanol and CO₂ gas under anaerobic conditions. The difference in temperature affects the level of consumption of reducing sugars during the fermentation process. Fermentation temperature is closely related to the metabolic and biochemistry activity of yeast cells during the fermentation process. Temperature greatly affects the enzymatic activity and turgidity of the yeast cell membrane [9].

Pretreatment process can reduce calcium levels on sugarcane molasses as a fermentation medium. Calcium has an antagonistic effect with magnesium. Magnesium is an essential cofactor that is needed in carrying out biochemical functions of yeast cells, an important cofactor of more than 300 enzymes, including enzymes that play a role in the glycolysis process [5]. A high level of calcium can inhibit biochemical functions of yeast cells [10].

3.5. Ethanol Content

After 48 hours fermentation, the fermented liquid is then distilled with a simple method distillation. Distilled of fermented liquid is measured using a density meter. The ethanol content of fermentation results obtained from calculations using the formula \( V_1 \times M_1 = V_2 \times M_2 \). Figure 1 shows the results of ethanol content in various treatments.

![Figure 1. Ethanol content from various treatments](image)

Ethanol content from various treatments ranges between 7.29 - 8.34%. The results of analysis of variance (ANOVA) showed sugarcane molasses fermentation medium that given pretreatment H₂SO₄ and fermentation temperature had a significant effect on the ethanol content of fermentation. The highest ethanol content was found in sugarcane molasses which were given pretreatment and 32 °C fermentation temperature as 8.31%.

Fermentation temperature is closely related to the activity of enzymes that play a role in cell metabolism. The higher fermentation temperature will affect the end of fermentation too quickly, which will cause the fermentation incomplete and produce low levels of ethanol [11]. Yeast cell viability will also decrease with increasing temperature [12]. This decrease occurs because the accumulation of intracellular ethanol in the cell is toxic to cells and changes the structure of the membrane, causing a decrease in cell membrane functionality [12].
3.6. Ethanol Yield
Ethanol yield from fermentation process was in the range of 53.38 - 68.67%. The higher the ethanol yield obtained, the fermentation process becomes more efficient. Table 5 shows the ethanol yield from various treatments.

| Treatment          | Ethanol yield (%) |
|--------------------|-------------------|
| H$_2$SO$_4$ pre-treatment 29 °C ± 1 °C | 64.71 ± 3.96 |
| Without pre-treatment 29 °C ± 1 °C | 54.86 ± 2.91 |
| H$_2$SO$_4$ pre-treatment 32 °C ± 1 °C | 68.67 ± 0.78 |
| Without pre-treatment 32 °C ± 1 °C | 55.33 ± 3.61 |
| H$_2$SO$_4$ pre-treatment 35 °C ± 1 °C | 59.37 ± 3.90 |
| Without pre-treatment 35 °C ± 1 °C | 53.38 ± 1.10 |

Note: The number after ± is the standard deviation value

Ethanol yield obtained in this study is higher than the theoretical yield. According to Harrison [13], maximum yield of ethanol by yeast cells is 0.51 g ethanol / g glucose or 51% which is calculated by stoichiometric reaction. Ethanol yield can be approximately 92-95% higher than the theoretical yield because ATP produced in the cell glycolysis process is not only used for cell growth and propagation, but also for other activities, such as maintaining cells from extreme conditions (inhibition ethanol and nutritional deficiency conditions) [14]. While according to Borzani and Jurkiewicz [15], there are two assumptions that can explain why the yield of ethanol can be higher than the theoretical yield. The first assumption is that there is a temporary ethanol accumulation in yeast cells which diffuses into the medium. The second assumption is the transformation of sugar into extracellular compounds that are not detected at the beginning of the fermentation process, which will then be fermented into ethanol.

3.7. Best treatment
Multiple Attribute Test was used to determine the best treatment for bioethanol production in this research. Based on Multiple Attribute Test, the best treatment obtained from sugarcane molasses medium that given H$_2$SO$_4$ pre-treatment and fermentation temperature of 32°C with total decrease value of total soluble solids (% brix) of 10.9 % brix, total decrease sugar of 12.15%, reducing sugar consumption of 57.21 g/L, ethanol content of 8.30 % and yield ethanol of 68.67 %.

4. Conclusion
The results showed sulfuric acid pre-treatment process and the fermentation temperature give significant effect on bioethanol production from sugarcane molasses medium by using instant dry yeast Saccharomyces cerevisiae. Based on Multiple Attribute Test, the best treatment obtained from sugarcane molasses medium that given H$_2$SO$_4$ pre-treatment and fermentation temperature of 32°C with total decrease value of total soluble solids (% brix) of 10.9 % brix, total decrease sugar of 12.15%, reducing sugar consumption of 57.21 g/L, ethanol content of 8.30 % and yield ethanol of 68.67 %.

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