Effect H$_2$SO$_4$ and Zymomonas mobilis concentration to bioethanol production by bintaro fruit (cerbera manghas) substrate

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Abstract. Bintaro fruit (Cerbera manghas) contains 36.945 of cellulose and 38% of lignin which is potential as a source of raw material for making bioethanol. The purpose of this research is to know the effectiveness of formula in producing bioethanol by the process of bintaro. The methods used in this research are pretreatment, delignification, hydrolysis, fermentation, and distillation. Pretreatment was performed by subcooling the substrate at 80°C followed by milling it up to 60 mesh. Delignification was performed by soaking the substrate in 100°C of 1N NaOH and 1 barr for 30 minutes. Hydrolysis was carried out with sulfuric acid catalyst (H2SO4) variations of 5.5%, 6.0%, and 6.5% at 120°C for 60 minutes. Fermentation with Zymomonas in variation of 1%, 3%, and 5% at room temperature for 3 days. The fermented filtrate was distilled at 73°C to obtain ethanol and tested the levels of ethanol with chromatography gas. The results showed that the highest levels of ethanol is 9.977% resulted from 6.5% of sulfuric acid hydrolysis and 5% of Zymomonas mobilis fermentation. The high levels of ethanol is supported by qualitative test result of the presence of reducing sugars with fehling yielding of 7002 ppm red brick tested quantitatively by nelson somogyi method. In conclusion, the level of bioethanol produced is directly proportional to the increased concentration of sulfuric acid and Zymomonas mobilis is used.

1. Introduction
Bintaro fruit (Cerbera manghas) is a mangrove plant that grows in abundance in tropical areas of Indonesia, and it has not been used maximally. Bintaro fruit is a fleshy stone fruit with a coconut-like lignocellulose fiber. The lignocellulose concentration found in Bintaro fruit consisted of 36.945% cellulose and 38% lignin [1]. The cellulose content of bintaro fruit is potential to serve as a substrate for the production of bioethanol through a series of processes starting from pretreatment, delignification, hydrolysis, fermentation, and distillation.

Research on the hydrolysis of cellulose into glucose was once performed by [2] using ultra-high temperatures and vapor pressure explosions with a percentage of 67.7% glucose yield at 60 atm for 1 minute), but the method is less practical. Cellulose hydrolysis can also be carried out with acids by [3] using a heterogeneous catalyst and cellulose pretreatment with a precipitation method resulting in 50% glucose. This method is done at high temperatures (140°C) so it is not practical enough.

Alkaline delignification (NaOH 1N) at 100°C, pressure of 1 bar, and 30 minutes of holding time was able to remove lignin in the lignocellulose arrangement so that hydrolysis was optimal [4]. [5]
his research to pretreatment swelling NaOH and HCl regeneration facilitate the process of depolymerizing cellulose into glucose. This is due to the amorphous hydrogel formed so that the structure is tenuous and easier to be hydrolyzed. Based on these facts, NaOH can be used for the preparation of cellulose from bintaro fruit. While the process of cellulose hydrolysis using HCl need to be replaced with H$_2$SO$_4$ because waste of HCl solution is difficult to degrade.

The highest glucose obtained by cellulose hydrolysis process from alfukate seeds using H$_2$SO$_4$ 6.0% at 120°C for 60 minutes in the autoclave [6]. The concentration of H$_2$SO$_4$ used is not specific and in the process does not use delignification. Therefore, in this research, the specification of H$_2$SO$_4$ concentration is used to obtain optimal condition.

[7] has fermented using *Saccharomyces cerevisiae* from cotton waste through pretreatment process first. *Saccharomyces cerevisiae* mushroom in this study was only able to produce 0.900g/L of ethanol. Other research on bioethanol is reported by [8] that fermentation can be performed using *Z. mobilis* bacteria and capable of producing a larger bioethanol than ethanol paper sludge for 48 hours of incubation. The research of bintaro fruit processing into bioethanol has been done by [9] with enzymatic hydrolysis and fermentation methods using *Saccharomyces cerevisiae*. The resulting bioethanol is relatively small at only 0.538% and is not proportional to the price of enzyme catalysts for relatively expensive hydrolysis. Therefore, *Zymomonas mobilis* bacteria potentially produce bioethanol from bintaro fruit with higher levels. The objectives of this study were (1) to know the effect of variation of concentration of sulfuric acid (H$_2$SO$_4$) solution to the glucose produced in cellulose hydrolysis of bintaro fruit, and (2) to know the effect of variation of *Zymomonas mobilis* concentration on bioethanol content of glucose fermentation process.

2. Methodology

2.1. Pretreatment

The initial stage of the research included soaked bintaro fruit weighing and cutting into small pieces. Subsequently, a physical pretreatment was done through drying at 80°C for three days, continued by dried bintaro fruit weighing and its water content measurement, ended with milling to obtain 40-60 mesh bintaro fruit powders.

2.2. Delignification

Substrate (bintaro fruit powders) immersion was done in 1L 1N NaOH at 100°C and 1 bar pressure for 30-minutes holding time in an autoclave in accordance with the research conducted previously by [4]. On the completion of the delignification process, the substrate was rinsed for several times using distilled water in order to neutralize the pH of the solution. Then, the process was followed by substrate filtration using filter paper to be then drained in the oven at 60°C for three days [10].

2.3. Hydrolysis

After drained in the oven during the delignification process, the substrate underwent a hydrolysis with sulfuric acid (H$_2$SO$_4$) catalyst varied to 5.5%, 6.0%, and 6.5% made by diluting 98% concentrated sulfuric acid in distilled water. 10.0g of the substrate was weighed and put into 3 Erlenmeyer flasks containing 100.0mL of sulfuric acid with the variations of 5.5%, 6.0%, and 6.5%. Next, the Erlenmeyer flasks were put into an autoclave to be then heated at 120°C for 60 minutes [9]. After 60 minutes of the hydrolysis process, the Erlenmeyer flasks were taken from the autoclave, and they were cooled down by being soaked in water. Afterwards, a filtration using filter paper was done to obtain the filtrate.

2.4. Fermentation

The pH of the filtrate needed to be increased until it reached 4.5 by adding 5 M NaOH solution via a universal indicator [11]. *Zymomonas mobilis* bacterium was then added into the 5.5%, 6.0%, and 6.5% filtrate with variations of 1%, 3%, and 5%. The addition of *Zymomonas mobilis* bacterium into 90.1mL filtrate was done in 0.901mL, 2.703mL, and 4.505mL respectively. The holding time for the fermentation
using *Zymomonas mobilis* bacterium was three days at room temperature [12]. After the 3-day fermentation, the filtrate was heated at 50°C to kill the *Zymomonas mobilis* bacterium.

### 2.5. Distillation

Distillation was done after the bacterium in the filtrate was eliminated. Heating filtrate at temperature was managed to be 74°C.

### 3. Research Findings and Discussion

#### 3.1. Identification of Water Content in Substrate

The analysis results of water content of observed bintaro fruit substrate in reddish green color are presented below.

| (a) No | (b) Bintaro Fruit Weight | (c) Fresh Weight (g) | (d) Dry Weight (g) |
|--------|----------------------------|----------------------|-------------------|
| (e) 1  | (f) 160.146                | (g) 41.336           |
| (h) 2  | (i) 158.375                | (j) 40.660           |
| (k) 3  | (l) 160.630                | (m) 41.448           |
| (n) Average | (o) 159.717 | (p) 41.148 |

Based on the above data, the average fresh weight of the bintaro fruit was 159.717g and the average dry weight after baked for three days was 41.148g. The water content in the fresh bintaro fruit was 74.24 %, which was obtained from the following equation.

#### 3.2. Filtrate Visualization by Fehling Test

The visualization of hydrolysate using Fehling test is given below.

![Fehling test results on sulfuric acid concentration of (a) 5.5%; (b) 6.0%; (c) 6.5%](image)

Figure 1 shows the occurrence of yellow or red cuprous oxide sediment, which indicated the presence of reducing sugar in the hydrolysate. Out of the three used concentrations, there was not any significant different red color among the 5.5%, 6.0%, and 6.5% samples. The sediment indicated that the polysaccharide conversion or breakdown process becoming reducing sugar was successful. The fehling test observation revealed that a high sulfuric acid concentration used at a high temperature would increase the production of the reducing sugar concentration in a red color.

#### 3.3. Reducing Sugar Concentration by Nelson Somogyi Test

Figure 2 shows that the higher sulfuric acid concentration, the higher the produced glucose concentration. In this case, the sulfuric acid serves as a catalyst that is aimed to accelerate the hydrolysis reaction. According to [13], a high number of catalysts shall result in a quick hydrolysis reaction. [14] revealed that the increasing acid concentration in hydrolysis process could also lead to a degradation of glucose and other sugar compounds becoming hydroxyl methyl furfural (HMF) compound and furfural compound, which eventually forms formic acid. However, as seen from the graph above, the acid concentration that increased up to 6.5% had not caused the glucose and other...
sugar compounds to degrade into hydroxyl methyl furfural (HMF) compound. Therefore, it can be concluded that 6.5% sugar concentration is still open for enhancement.

![Figure 2. Comparison of H2SO4 Concentration and Reducing Sugar Concentration.](image)

The above graph presents that there was 7002 ppm reducing sugar concentration resulted from 6.5% sulfuric acid concentration. The higher the sulfuric acid concentration, the more the number of H+ bonding the hydroxyl group on the polysaccharide, which results in the release of interchain bonding that forms monomers, especially in the shape of monosaccharide. The increase of the sulfuric acid concentration lead to the breakdown of the polysaccharide became a high number of monosaccharide and disaccharide. The monosaccharide that was formed to reduce the cupric oxide would be detected by Uv-Vis after reacted to arsenomolybdate and formed dark blue-colored molybdenum. Such sample was then used for fermentation.

### 3.4. Gas Chromatography Test Results

The data analysis from the gas chromatography is presented in Table 2 below.

| No | Name                     | Weight Area | Ethanol | Ratio | Content (%) |
|----|-------------------------|-------------|---------|-------|-------------|
| 1. | 5.5% H2SO4 + 1% Z. mobilis | 0.0198, 0.2076 | 13,264.81, 6,696,686.82 | 0.002 | 2.284       |
| 2. | 5.5% H2SO4 + 3% Z. mobilis | 0.1036, 0.229 | 184,499.16, 8,114,377.92 | 0.025 | 5.380       |
| 3. | 5.5% H2SO4 + 5% Z. mobilis | 0.4933, 0.2367 | 1,273,772.80, 8,438,722.22 | 0.166 | 7.980       |
| 4. | 6.0% H2SO4 + 1% Z. mobilis | 0.4966, 0.2407 | 514,893.53, 8,819,420.15 | 0.064 | 3.112       |
| 5. | 6.0% H2SO4 + 3% Z. mobilis | 0.0989, 0.2074 | 54,714.89, 1,870,529.14 | 0.032 | 6.746       |
| 6. | 6.0% H2SO4 + 5% Z. mobilis | 0.4827, 0.2394 | 1,382,787.81, 8,087,229.88 | 0.188 | 9.326       |
| 7. | 6.5% H2SO4 + 1% Z. mobilis | 0.0424, 0.0079 | 1,049,193.92, 4,719,776.43 | 0.244 | 4.555       |
| 8. | 6.5% H2SO4 + 3% Z. mobilis | 0.3438, 0.2397 | 1,117,349.23, 9,580,964.72 | 0.128 | 8.942       |
| 9. | 6.5% H2SO4 + 5% Z. mobilis | 0.0499, 0.2395 | 150,034.92, 7,937,617.42 | 0.021 | 9.977       |

The gas chromatography test results were analyzed using double anova preceded by data normality test via Kolmogorov - Smirnov normality test. The normality test showed that the obtained data had a normal distribution with a p value of 0.137. The data with normal contribution was then statistically tested using double ANOVA, and the results presented that the increase of H2SO4 concentration and the use of Zymomonas mobilis affect the obtained amount of bioethanol. It showed that the increase of the sulfuric acid concentration and Zymomonas mobilis is directly proportional to the increasing production of the ethanol concentration. The high concentration of sulfuric acid will increase the capability to produce glucose from cellulose. The high concentration of glucose functions as the main source of nutrition for the microorganism, which resulted in more glucose to be converted into ethanol. From this research, it is known that 6.5% H2SO4 and 5% Zymomonas mobilis are the most efficient concentrations to produce bioethanol.
The ANOVA test on the interaction between the increase of H$_2$SO$_4$ concentration and Zymomonas mobilis used did not affect the production of bioethanol. It showed that the increase of the H$_2$SO$_4$ concentration and Zymomonas mobilis are not directly proportional to the production of bioethanol. Such ANOVA analysis results can be seen from the increase of 3 – 5% concentration (sample 8 and 9) which was relatively low, namely 1.035% while the 1-3 % concentration (sample 7 and 8) had 4.387% difference. Therefore, it can be concluded that on the 3–5% concentration increase, the phase of the microorganism growth developed and they started to look for a food source (glucose) so that the death phase became faster and resulted in the low improvement of the ethanol concentration in sample 8 and 9.

The increase of acid concentration in the hydrolysis process also could cause a degradation of glucose and other sugar compounds become hydroxyl methyl furfural (HMF) and furfural, which shall form formic acid [14]. The first cause of the high concentration of ethanol produced in this research was the absence of sterilization on the hydrolysate that was going to be fermented via autoclave, since it would form an inhibiting compound that prevents the performance of the microorganism during the fermentation [17]. Second, the microorganism used in this research has better performance than Saccharomyces cerevisiae in producing bioethanol. Such fact is in line with the research conducted by [18] and [19] stating that the concentration of ethanol results from the Zymomonas mobilis fermentation is higher than that from the Saccharomyces cerevisiae fermentation.

### 3.5. Comparison of Research Result

This research is the second research to process the bintaro fruit into bioethanol. Previously [9] have conducted similar research. Therefore, the following is a detailed discussion of the comparative research results in terms of economic aspects, effectiveness, and efficiency of research.

**Table 3.** Comparative research in terms of effectiveness of glucose being converted.

| Substrate Concentration | Hydrolyzate | Fermentation Glucose Level (ppm) | Ethanol Yield |
|-------------------------|------------|---------------------------------|---------------|
| 100 g/L                 | No         | Before 8604.9 After 1870,185    | 0.538%        |
|                         |            | Converted 6734,185              |               |

(source: [9])

| Substrate Concentration | Hydrolyzate | Fermentation Glucose Level (ppm) | Ethanol Yield |
|-------------------------|------------|---------------------------------|---------------|
| 100 g/L                 | No         | Before 7002 After 109.9         | 0.62%         |
|                         |            | Converted 6892,1                |               |

**Table 4.** Comparison of research in terms of efficiency of research implementation

| No. | Substance of Study | Iman and Handoko, 2011 | Santos et al., 2017 |
|-----|--------------------|-------------------------|---------------------|
| 1   | Preparation        | 3 days                  | 1 days              |
|     | Delignification    | -                       | 30 minutes          |
|     | Hidrolysis         | 3 days                  | 1 hours             |
|     | Fermentation       | 3 days                  | 3 days              |
|     | Distillation       | 2 hours                 | 2 hours             |
|     | **TOTAL (Time)**   | 9 days dan 2 hours       | 4 days and 3,5 hours |

**Table 5.** Comparative research in terms of economic value of research implementation

| No. | Substance of Study | Iman and Handoko, 2011 | Santos et al., 2017 |
|-----|--------------------|-------------------------|---------------------|
| 1   | Delignification    | -                       | 4 g (80,- IDR)      |
|     | Hidrolysis         | 5 mL enzim cellusoft L (75.000 IDR) | 6,63 mL H$_2$SO$_4$ (104,- IDR) |
|     | Fermentation       | 5 g S. cereviceae (1.863,65 IDR) | 4,5 mL Z. mobilis (1.750,- IDR) |
|     | **TOTAL (Value of Substrate Processing)** | 76.863,65 IDR | 1.934 IDR |
Holistic conclusion obtained from the above three comparisons is this research is superior compared to [9]. It also indicates that this research is very feasible to be implemented massivly to process the source of cellulose substrate in Indonesia to make it bioethanol.

4. Conclusion

From the research findings, it is known that bintaro fruit has 74.24% water content. Under the Nelson-Somogyi quantitative test, the identification of the highest reducing sugar concentration was 7002 ppm in 6.5% sulfuric acid variation. Gas chromatography showed that the highest ethanol concentration was obtained from the 6.5% variation of sulfuric acid and 5% Zymomonas mobilis with a percentage of 9.977%. The results of this study also revealed a directly proportional relationship between the increase of the sulfuric acid concentration as a catalyst and the Zymomonas mobilis microorganism in the production of ethanol. The ethanol yield produced from 1 kg of fresh bintaro fruit was 9.0 mL/kg of fruits. The results of comparative observation with previous research indicate that this research has advantages in terms of effectiveness, efficiency, and economic value of implementation formula.

The suggestions are to develop the current research by increasing the sulfuric acid concentration for the hydrolysis process and to specify the Zymomonas mobilis content in the range of 3-5%.

5. References

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