The Effect of Sugar Concentration and Time for Nypa Sap Fermentation into Acetic Acid using *Acetobacter pasteurianus*

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Abstract. Acetic acid is widely used in cosmetics, manufacturing chemicals, food additives, or in some countries used as drinks. So that the needs for acetic acid increase every year. Acetic acid can be produced by fermentation using raw nypa sap containing sucrose, glucose and fructose. Factors affecting the fermentation process are the substrate sugar concentration and fermentation time. The purpose of this study was to determine the effect of increasing sugar concentration, the effect of fermentation time on bioethanol concentration, and the effect of fermentation time on acetic acid concentration. The fermentation process was carried out in two stages, namely Bioethanol Fermentation using *S. cerevisiae* with variations in sugar concentration of 149 g/L and 202 g/L and fermentation time of 1, 3 and 5 days. The bioethanol produced was continued by the acetic acid fermentation process using *A. pasteurianus* with variations of the fermentation time of 1, 3, 5, 7 and 9 days. The maximum conditions for acetic acid production were 202 g/L sugar concentration, 39.47 g/L bioethanol substrate, and the 9th day fermentation, at acetic acid concentration of 36.71 g/L and yield 71.37%.

1. Introduction

Indonesia is one of the countries that have the largest nypa plant in the world, with the area of nypa plants, which is 10% of tidal areas of 7 million hectares [1]. Nypa is a member of the palmae plant, growing along rivers that are affected by tides and is grouped in mangrove forest ecosystem. Nypa grows naturally and from ecological aspects is useful to protect the shoreline from the process of abrasion by sea waves. Non-wood forest products from nypa are sap which is a sweet liquid resulting from bunches which are known to be processed into acetic acid using a fermentation process [2]. Nypa sap can produce 20 tons/hectare of sap which is twice as large as sugarcane. Nypa sap has the nature of being easy to become acidic due to the fermentation process by *S. cerevisiae*. Where *S. cerevisiae* can convert sugar into alcohol, then it becomes acetic acid by using bacteria [1].

Acetic acid or vinegar is divided into two types, there are synthesis vinegar and synthetic vinegar. Synthesis vinegar is produced directly from synthetic vinegar with the addition of water and caramel, while synthetic vinegar is produced from fruit juices such as apples, oranges, grapes, oranges, and sugar-containing ingredients using the fermentation process. Acetic acid is currently consumed in large quantities throughout the world. Acetic acid is used as a flavoring agent, food preservative, and in some countries as well as drinks [3].

The fermentation process is a conventional process of initial processing technology, which is better known as the development of the role of further technology, both primary, secondary, and bioconversion metabolism to manipulate the overhaul of the composition of the structure of cell tissue structures [4]. The fermentation process is a process. The production of acetic acid through a two-
stage fermentation process. The first fermentation process of the formation of bioethanol using *S. Cerevisiae* [5].

\[
C_6H_{12}O_6 \xrightarrow{\text{Yeast}} 2C_2H_5OH + 2CO_2
\]  

(1)

The second stage of fermentation is the formation of acetic acid using bacteria *A. pasteurianus* [6]. Acetic acid bacteria are used for aerobic fermentation, by oxidizing bioethanol to acetic acid. Bioethanol reacts with oxygen to produce water and acetic acid. Acetaldehyde acts as an intermediary in the formation of acetic acid [3].

\[
C_2H_5OH + \frac{1}{2}O_2 \rightarrow CH_3COOH + H_2O
\]  

(2)

Factors influencing the fermentation process are the culture potential in producing acetic acid, its resistance to ethanol as a substrate or acetic acid as a product, and the process conditions which include substrate concentration, initial pH of media, nutrients, temperature, fermentation time, oxygen, and stirring. Fermentation can occur due to microbial activity that causes fermentation on suitable organic substrates [7]. The objective of this study was to evaluate the effect of increasing sugar concentration, the effect of fermentation time on bioethanol concentration, and the effect of fermentation time on acetic acid concentration.

2. Methodology

2.1. Materials and Tools.

Materials used were nypa sap, *S. cerevisiae* Y-613 and *A. pasteurianus* B-377, GYP, PDA, benedict reagents, aquades, NaOH, NPK, urea, K$_2$HPO$_4$, MgSO$_4$.7H$_2$O, PP indicator, Nelson-Somogyi solution. The tools used were autoclaves, 2-liter reactors, magnetic stirer, shakers, incubators, erlenmeyers, beakers, flasks, drop pipettes, volume pipettes, petri dishes, bunsen needles, pH meters, volume pipette, titration device, evaporator circuit.

2.2. Preparation of Raw Materials

The raw material used in this research is nypa sap. In the process of tapping nypa sap, nypa bunches must be cleaning, then bunches are beaten so that the pores are open and then shaken so that the sap is swiftly removed, then cut the tip of the bunch and collected using a bottle. To get pure sap, then in the process of tapping there is no effort to get garbage, dirt that enters.

2.3. Pretreatment of Raw Materials

Nyra sap must be prepared before being used as a fermentation medium. Nyra sap is heated first. The Purpose of heating process aims to reduce the water content contained in the nypa sap so that the concentration of sugar increases. The heating process is carried out by varying the concentration of 25% (v/v) nypa sap. The tools used in the producing process, inoculum preparation, and fermentation process must first be sterilized to prevent contamination that can affect the results fermentation. Sterilization was carried out at 121°C for 15 minutes using an autoclave [8].

2.4. Bioethanol Fermentation

Inoculum *S. cerevisiae*. Inoculum *S. cerevisiae* aims to adapt cells yeast and bacteria to the fermentation media. With the adaptation process, the slow phase as the initial stage of fermentation has been bypassed. Inoculum volume in this research is 10% of the total substrate medium.

Fermenters used are 2-liter fermerter. Fermentation is carried out at room temperature and pH 5 is maintained at a stirring speed of 200 rpm. The fermentation process is carried out by the addition of 0.4 g/L urea and 0.5 g/L NPK as nutrition with a media volume of 1800 ml inserted into the fermenter.
Then all medium sterilized in the autoclave, cooled at room temperature, and add 200 ml of inoculum into the fermentation medium. Samples are taken at 1, 3, and 5 fermentation days.

2.5. Acetic Acid Fermentation
The volume of inoculum _A. pasteurianus_ used in this study was 10% from the first fermentation. Then put it into an erlenmeyer and K$_2$HPO$_4$ 3.3 g/L and MgSO$_4$.7H$_2$O 1.1 g/L as nutrition. Then add 5 ml of GYP into the test tube and _A. pasteurianus_ for 2 ose needles, and incubate for 24 hours. After incubation, insert it into the inoculum medium then stir using a shaker for 24 hours at room temperature. After that, the inoculum can be used for the next process [6, 9].

Fermenter process used is 500 ml erlenmeyer. Fermentation is carried out at room temperature and initial pH 6. The fermentation process is carried out by adding nutrients K$_2$HPO$_4$ 3.3 g/L and MgSO$_4$.7H$_2$O 1.1 g/L. Then add the inoculum _A.pasteurianus_. Samples are taken at 1, 3, 5, 7, and 9 fermentation days.

3. Results and Discussion

3.1. Pretreatment of Nypa Sap with the Heating Process.
The raw material used in this research is nypa sap. In addition to glucose and fructose in nypa sap there is also sucrose. According to Tamunaidu [10] sucrose contained in pure nypa sap is 97.5 g/L. In the fermentation process, glucose fermented by yeast into bioethanol and CO$_2$ [11]. So it needs preliminary treatment to break down sucrose into glucose and fructose. Test results with Benedict solution is shown in Figure 1 below.

![Figure 1. Test Results with Benedict Solution](image)

In Figure 1 shows that pure nypa sap produces a green color. It was indicate that in pure nypa sap contain a higher sucrose than glucose and fructose. Whereas in nypa sap after the evaporation process 15% (v/v) produces yellow, evaporation of 20% (v/v) produces orange color and 25% (v/v) evaporation of brick red. The sugar containing aldehyde groups or free ketones will reduce Cu$^{2+}$ ions in the alkaline atmosphere, to Cu$^{2+}$, which settles as Cu$_2$O (cuprous oxide) brick red in Benedict test [12].

3.2. Bioethanol Concentration.
The effect of fermentation time on bioethanol concentration is shown in Figure 2 below.
Increasing sugar concentration and fermentation time had a very significant effect on the bioethanol concentration obtained. Figure 2 shows that the levels of bioethanol increased at sugar concentration of 149 g/L and 202 g/L. This shows that increasing the sugar concentration in the fermentation process can increase the bioethanol concentration. According to Simanjuntak [13], the maximum sugar concentration used for the fermentation medium is 200 g/L. In sugar concentration of 149 g/L and 202 g/L, the bioethanol concentration produced increase on the 1st day of the 3rd day. This is caused in this phase, *S. cerevisiae* uses substrate as a nutrient for the growth so that it can multiply rapidly and it causes the growth rate of *S. cerevisiae* increase so the concentration of bioethanol produced increases. This is proven by decreasing the concentration of sugar at each time of fermentation where the initial sugar concentration of 149 g/L becomes 61.15 g/L and 202 g/L becomes 57.09 g/L. While the concentration of bioethanol in each variation of the concentration of sugar produced during the 5th day fermentation begins to decrease. At each treatment in sugar concentration decreased bioethanol concentration on the 5th day. This explains that *S. cerevisiae* have passed exponential phase on that day. However, after the optimum conditions are reached, on the 5th day of fermentation the bioethanol concentration decrease. This is because the nutrients in the fermentation medium are getting smaller, besides the decrease in the concentration of bioethanol is influenced by the further reaction of the change in bioethanol to acetic acid [8].

3.3. Acetic Acid Fermentation.
The effect of fermentation time on acetic acid concentration is shown in Figure 3 below. In Figure 3 shows the relation between the fermentation time of acetic acid to the acetic acid concentration and sugar concentration. From the figure 3, it can be seen that acetic acid has formed from day 1 to day 9 fermentation. Figure 3 (a) and (b) show that the maximum acetic acid concentration was obtained at a bioethanol concentration of 39.47 g/L at a sugar concentration of 149 g/L that is 21.62 g/L and a sugar concentration of 202 g/L at a bioethanol concentration of 39.47 g/L on the 9th day that is 36.71 g/L. The maximum acetic acid concentration was obtained at a bioethanol concentration of 39.47 g/L on the 3rd day of bioethanol fermentation from each preliminary treatment. This is because *A. pasteurianus* has the optimum bioethanol concentration for use as a fermentation substrate. According to Wibowo [8], it was happen a side reaction followed by the formation of acetic acid before the acetic acid fermentation process was carried out, so that the concentration of acetic acid produced was higher. The production of acetic acid is the result of the process of the activity of...
trapped cell enzymes and the support of the enzyme activity of the cells that escaped. In the process of acetic acid fermentation there are two enzymes involved, the enzyme alcohol dehydrogenase (ADH) and the aldehyde dehydrogenation enzyme (ALDH). ADH has a function as oxidation catalysts bioethanol into acetaldehyde by the following reaction:

\[
\text{C}_2\text{H}_5\text{OH} + \frac{1}{2} \text{O}_2 \xrightarrow{\text{ADH}} \text{CH}_3\text{CHO} + \text{H}_2\text{O}
\]

While the ALDH serves as an oxidation catalyst of acetaldehyde into acetic acid reaction as follows:

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**Figure 3** Effect of Fermentation Time on Acetic Acid Concentration

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Acetaldehyde is a compound produced from the oxidation of ethanol by the ADH enzyme. In the production of acetaldehyde by oxidizing ethanol to acetic acid, the oxidation process cannot be known, because the process takes place quickly then proceed with the oxidation of acetaldehyde to acetic acid with the ALDH enzyme. In Figure 3 (b) shows the highest concentration of acetic acid In this research obtained the highest acetic acid concentration at a bioethanol concentration of 39.47 g/L or 5% (v/v). According to Luwihana [6] ethanol 5% is the optimum concentration as a substrate for acetic acid fermentation, where the acetic acid production decreases in the use of ethanol greater than 5%. Ethanol can increase membrane permeability in Gram negative bacteria, so the diffusion of ethanol into cells will increase. An increase in the diffusion of ethanol will result in an increase in the production of acetic acid produced. The yield of acetic acid produced in this research is relatively lower than theoretically, from the lowest at 5.69% to the highest of 71.37%. theoretically 1 gram of ethanol will produce 1.3 grams of acetic acid. According to Gorie [14], in the process of fermentation of acetic acid that goes well the ethanol concentration which is oxidized to acetic acid is 95-98%, but in reality not all ethanol in the fermentation medium can be converted to acetic acid. This is because ethanol is also used by bacteria as a carbon source for cell growth and also evaporation that occurs during the fermentation process.

4. Conclusion
Nypa sap is used as a fermentation substart with sugar concentrations of 149 g/L and 202 g/L. The best results were obtained at a sugar concentration of 202 g/L, bioethanol concentration of 39.47 g/L with acetic acid concentration obtained on the 9th day fermentation of 36.71 g/L.

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