Effect of Nira (*Arenga pinnata Merr*) Tapping Tools on the Abudance and Identification of Microorganisms

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Abstract

Arenga trees are known to have economic value, especially its water, called nira. Nira contains sucrose with a neutral pH at the start of tapping, but after 4 hours, the quality of nira will decrease. This is due to the influence of air (aerobic), so that the presence of microbes in nira becomes abundant and causes the fermentation process to take place more quickly. The fermented nira is known as 'tuak', the traditional drink of Bataknese people. Batak people believe the benefits of drinking tuak for health. In this study, nira was collected using a simple tapping device (APUS) with the aim of minimizing contact between surrounding air and bunches. APUS was applied to Arenga bunches in Samosir. Nira from tapping with and without APUS will be observed for microbial abundance (its density). Furthermore, the microbes contained in nira will be identified based on their morphological characters (physically). Identification aims to determine the types of microbes that are present from a natural habitat. Next, the microbes are isolated and studied for their shape and properties, before the microbes are utilized further. The results of microscopic observations showed that the nira sample tapped without using APUS (sample A) had a higher and more turbid microbial density and showed non-uniform microbial size (from large to small, like powder), while nira sample tapped with APUS (sample B) showed almost uniform microbial size with cleaner looking spots. Furthermore, the microbes in sample B were isolated on specific media. Acquisition of colonies in the form of acetic acid bacteria (BAA), lactic acid bacteria (BAL), and yeast. BAA isolates showed a group of gram-negative bacteria that could not retain the crystal violet stain when rinsed with alcohol, because the peptidoglycan in gram-negative bacteria was thinner than gram-positive ones, while BAL isolates were the opposite. The measurement of the number of colonies was carried out using the TPC (Total Plate Count) method at 6 dilutions and obtained for BAL and yeast were 147 and 30 colonies respectively.

Keywords: *nira, APUS, microbes, observation, identification*

I. INTRODUCTION

The arenga tree is also known as the enau tree with the botanical name *Arenga pinnata merr* which belongs to the Aracaceae or palma family. The arenga tree is a large perennial plant with a height of up to 12 meters. The flowers of the aren palm tree grow on the segments of the trunk and resemble bunches with hanging strands of flowers [1]. The palm tree begins to produce nira water at the age of 12 years with the characteristics of a tree ready to be tapped, namely having a flower stalk of almost 3 meters, oily young leaves, large and sturdy flower stalks [2]. One palm tree can be tapped up to 4 bunches in a year and each bunch can be tapped within 3-5 months.

Fresh nira water that has just dripped tastes sweet, has a pH of around 7. Nira water contains sugar and can be a habitat for the growth of myroorganisms. Yeast and bacteria are contained in the nira water, which comes from the tree bunches and from the free air in the environment around the tree [3]. The air for living systems be a transport medium to distribute particle components, one of which is biological particles such as bacteria [4].

Tapping generally takes almost 12 hours, during which time the sap collection container and the ends of the bunches are exposed to the air. Exposing the juice to the air can accelerate the growth of microbes. This can cause the taste of the juice to change more quickly as the sugar in the juice breaks down more quickly. Also, more nira water is collected during the tapping process. The sweet taste of the nira will become sour and smell of alcohol. This is called tuak. Through this study, the first step was the use of a tapping device (APUS) that has been made to collect sap water to minimize its contact with environmental air. This minimization is expected to reduce microbial density during tapping. Furthermore, the microbes contained in the nira will be identified based on their morphological (physical) characteristics. Identification aims to determine the type of microbes present in a natural habitat. Furthermore, microbes are isolated and studied for their properties, before they are utilised further.

II. MATERIALS AND METHODS

A. Materials and equipment

Nira tapped with APUS as raw material. Media NA (Nutrient Agar), PYG (Peptone Yeast-Extract Glucose), MRSA (deMan, Rogosa and Sharpe Agar), PDA (Potato Dextrose Agar), and other supporting raw materials such as sodium chloride (Merck), glucose (97.5% purity; Merck), hydrogen peroxide (50% purity), and calcium carbonate.

The tools used were autoclave (HVA-110), incubator (TermoStable SIF-105), Genesys 30 UV-Visible Spectrophotometer (Thermo Fisher Scientific), Alcohol Refractometer (RA-600), universal pH indicator, and Binocular Biology Microscope (Eclipse E100 LED).

B. APUS assembly and application.

A simple APUS was assembled and applied for collecting nira from arenga tree in Samosir, North Sumatra Province. The simple APUS was attached to the end of the bunch, where nira drips. Rubber material was chosen as the tip of the APUS. It it easier for the tip to be attached to different diameters of the bunches. There are two chambers in the APUS, showed at Figure 1. First is a small chamber for temporary collection of nira before to second chamber. Second chamber, a jerry collected a larger volume of juice, also was installed valve for pH sampling. Sampling is performed every one hour. Between the first and second chambers, a connecting rubber tube is installed.

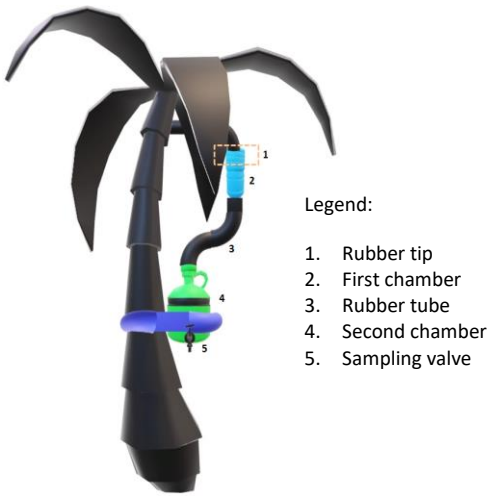


Figure 1. Tapping tool for nira (APUS)

C. Inoculation and microbial abundance observation.

Two samples of nira as object of observation were tapped with APUS (called **sample A**) and without APUS (called **sample B**) brought to Bioprocess Engineering Laboratory, Del Institute of Technology, using cooler box. Then, 0.01 ml each sample was inoculated in nutrient agar (NA) media. The inoculum was spread and incubated for 24 hours. After incubation, samples are observed of microbial abundances.

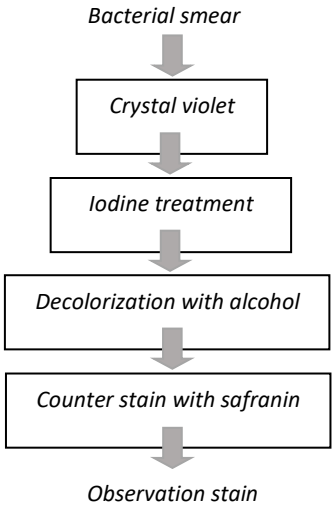


Figure 2. Bacterial gram stain procedure [5]

D. Isolation and identification of single colony.

Isolation is the process of separating one bacterium from its population in nature. Then, the next step is identification. Identification comprises of morphology, growth, and biochemical tests [6]. The isolation and identification process were carried out at the

Bioprocess Engineering Laboratory, Institut Teknologi Del.

a. Single Colony of Acetate Acid Bacteria (AAB)
1 mL of sample A was put into petri dish containing solid PYG media. After that, the incubation process was carried out for 48 hours at a temperature of 37°C. Lastly, AAB were observed its morphology, through bacterial gram staining. The staining technique follows the Hans Christian Gram procedure (Figure 2).

b. Single Colony of Lactic Acid Bacteria (LAB)
Isolation LAB was MRSA medium added with 1% $CaCO_3$. It aims to neutralize acid production by LAB. 1 ml of sample A is taken and put into 9 ml of 1% sodium chloride (NaCl) solution, and is called a 10^{-1} dilution. That mixture was then serially diluted (10^{-1} to 10^{-6}) for bacterial enumeration. For each dilution, 1 ml was taken and poured onto solid MRSA media, then evenly distributed using spread method. Then, it is incubated at 37°C for 48 hours. Lastly, LAB were observed its morphology, through bacterial gram staining.

b.1 Total plate count (TPC)
Counting of LAB colonies with TPC (Total Plate Count) method. The TPC formula is given in Equation 1 below:

$$\text{TPC} = \frac{\text{colonies form unit}}{\text{dilution factor}}; \left[\frac{\text{CFU}}{\text{ml}}\right] \quad \text{..... Equation 1}$$

b.2 Catalase test
In order to identify the activity of the catalase enzyme, hydrogen peroxide (H_2O_2) solution was used. Catalase testing is done by taking one oose of LAB suspension, placed it on the object glass then dripped 1-3 drops of H_2O_2. Next, it was observed the presence of gas bubbles.

c. Single Colony Yeast
The steps of isolating yeast follow the previous steps, but using PDA media. Then the total number of yeast colonies was counted with TPC.

c.1 Productivity yeast
250 mL of distilled water in an erlenmeyer was added with 2.5 g yeast extract and 25 g glucose, then homogenized it. Afterwards, the solution was sterilized in an autoclave. Next, into it was added yeast inoculum and incubated it at room temperature. Growth tests were carried out by measuring the cell concentration of yeast. Measurements were taken using a spectrophotometer at 660 nm. During growth, yeast will be tested for its productivity in forming alcohol.

III. Results and Discussions

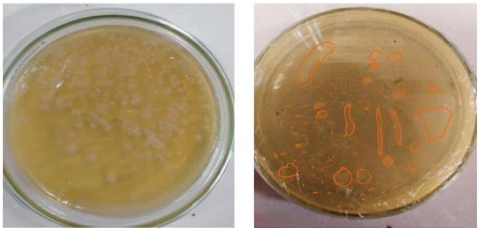
Nira water tapping was conducted in the morning and collected in the evening, 8 am till 5 pm. During the tapping, the p of nira was measured and shown in Table 1.

Table 1. pH measurement during taping

Time	pH Nira	
	Sample A (With APUS)	Sample B (Without APUS)
08.00	8	7
09.00	8	7
10.00	7	7
11.00	7	6
12.00	6	6
13.00	6	6
14.00	6	5
15.00	5	5
16.00	5	4
17.00	5	4

Observations in field while tapping showed that each litre of nira collected required around 115 minutes at the fastest and 123 minutes at the longest. The pH measurement (Table 1) showed a decrease. The quality of nira began to decline after 6 hours of tapping with an indication of pH to 5. However, the decline in pH of nira tapped without APUS occurred faster. Nira tapping usually lasts for 10-24 hours [7]. During tapping, there was an accumulation of nira in the APUS and caused natural fermentation to begin.

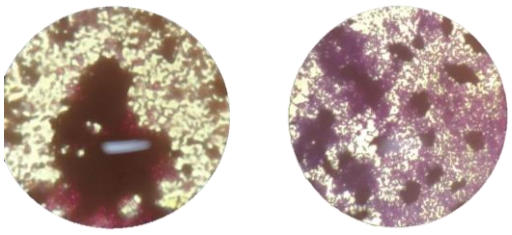
Two samples of nira were brought to Bioprocess Engineering Laboratory, inoculated on NA media, and incubated. Hereafter the samples are observed of microbial abundances after 24 hours incubation visually, shown in Figure 3.



(a) (b)

Figure 3. Visual observation of microbial population abundance in nira after incubation (a) with APUS and (b) without APUS

Isolation conducted on each specific media, then visual observation after gram staining for bacteria under microscope with 100x magnification.



(a) (b)

Figure 4. Microscopy visualization after gram-staining (a) AAB and (b) LAB

NA media is a common culture medium for growing various bacteria [8]. The form of NA is solid, which is made from mixture of natural materials such as meat extract and peptone, and uses agar as a solidifier. NA contains carbohydrates, namely galactams, which are not easily decomposed by microbes. On the other hand, meat extract and peptone are sources of protein, nitrogen, vitamins, and carbohydrates for microorganisms [9].

Two samples of nira that have been spread on NA and incubated for 24 hours, it can be seen that the microbial population is denser in nira tapped without APUS. As shown in Figure 3, the incubation results of microbes in nira tapped without APUS have a non-uniform size (from large to small size like powder).

A. Acetic acid bacteria (AAB) identification study

Acetic acid bacteria are gram-negative that grow aerobically with the cell shape is rod, and tolerant to acidic conditions and can grow at pH below 5 [10]. However, the cell form of AAB is not only found in rod, but also ellipsoidal, straight or curved, and can be found singly (monobacilli), in pairs (diplobacilli) or in chains [11]. In this study, isolation of AAB in PYG media and then staining. The staining result indicated that BAA could not retain the crystal violet smear (Figure 4) when rinsed with alcohol, because the peptidoglycan in gram-negative bacteria is thinner than gram-positive bacteria [12].

AAB is present in the nira as well as other microbes such as yeasts and LAB. Generally, the population of AAB is low, but will increase when the materials are spoiled or decomposed. AAB produces acetic acid through two metabolic stages, alcohol dehydrogenase and acetaldehyde dehydrogenase, with the presence of oxygen (obligate aerobic). When the material contacts air, BAA will provoke a quick change in taste to sour, and at high concentrations will create a bitter sour taste and vinegar-like aroma. The concentration of acetic acid, as in wine, is not desired to exceed 0.4-0.5 g/L [13].

B. Lactic acid bacteria (LAB) identification study.

Nira has been reported as an authentic source of LAB present [14]. As in this study, LAB isolates have been obtained from nira by isolating on MRSA media. Furthermore, the isolates were gram stained and counted the number of colonies using the TPC (Total Plate Count) method. The staining results (Figure 4) showed that LAB is a gram-positive bacterial group, with rod and round cell forms. Afterwards, the measurement of the number of LAB colonies using the TPC method has a requisite that the number of growing colonies does not exceed 300 colonies (TMTC category, too many colonies to count). The number of LAB colonies from TPC with 6 times dilution was 147.

The LAB characterization through catalase test. The results showed that LAB was unable to produce catalase enzyme, which means catalase negative, because no air bubbles were formed when the isolate was tested with H_2O_2 solution. According to the criteria, LAB will not produce catalase enzyme (catalase negative) [15]. The catalase that is not produced can increase the sensitivity of LAB to oxygen [16].

LAB is classified mainly into two categories based on its metabolism, namely homofermentative which uses the glycolysis pathway and heterofermentative which uses the pentose phosphate pathway or called phosphoketolase. Homofermentative *lactobacilli* are relatively scarce in the brewing process, the most dominant are facultative heterofermentative *lactobacilli* (FHE) [17].

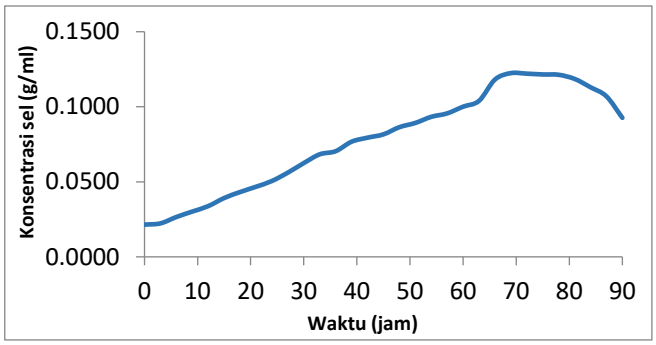
LAB do not require oxygen, but they will certainly not die in the presence of oxygen. Obligate homofermentative LAB (**OHO**) ferment hexose only (pentose and gluconate not) and almost exclusively to lactic acid via the glycolysis pathway. Facultative heterofermentative LAB (**FHE**) ferment hexose to lactic acid via glycolysis, and are also able to degrade pentose and gluconate (with limited sugars present) via the induced phosphoketolase pathway to produce acetic acid, ethanol and formic acid. At last, obligate heterofermentative LAB (**OHE**) possess FDB aldolases, but not phosphoketolases, which metabolize pentoses and hexoses exclusively via the

phosphogluconate pathway to produce lactic acid, ethanol and CO_2 [18].

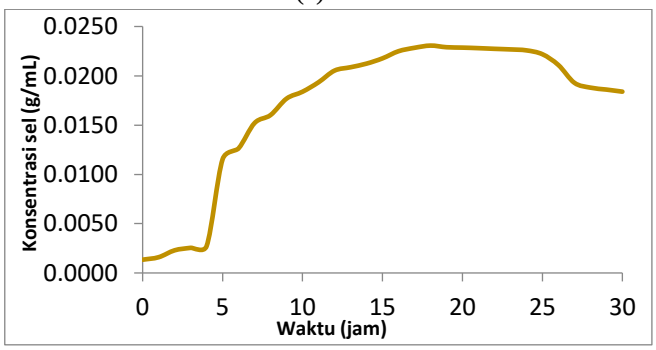
C. Yeast identification study.

Yeasts are unicellular fungi, which are present in a large number of environments, and have a high species diversity. The diversity of environments where yeast can be found is closely related to the varied carbon sources, such as glucose, fructose, galactose, or mannose [19]. *Saccharomyces* species, particularly *S.cerevisiae*, are commonly known and used in the production of fermented products that can be consumed by humans. In addition, the morphology of *saccharomyces* species is spherical or ellipsoidal, which depends on the growth phase and cultivation conditions [20].

The number of yeast colonies from TPC with 6 times dilution was 30. It has a lower amount than LAB colonies. This is also evidenced by the growth time of yeast which is three times longer than LAB (Figure 5). The growth phase of yeast until death is 90 hours while LAB needs 30 hours.



(a). Yeast



(b). LAB

Figure 5. Growth curve

Generally, yeast (*S.cerevisiae*) grows well at temperatures of 20-30°C, pH 4.5-6.5 and under facultative anaerobic conditions. Yeast growth and alcohol production are closely related. Yeast productivity was measured through alcohol formed during growth (Figure 6). Alcohol is produced as the cell attempts to maintain its redox balance and make enough ATP for continued growth [21]. Ultimately, sugar is used anaerobically by yeast as an electron donor, electron acceptor, and carbon source to produce alcohol and carbon dioxide (CO_2).

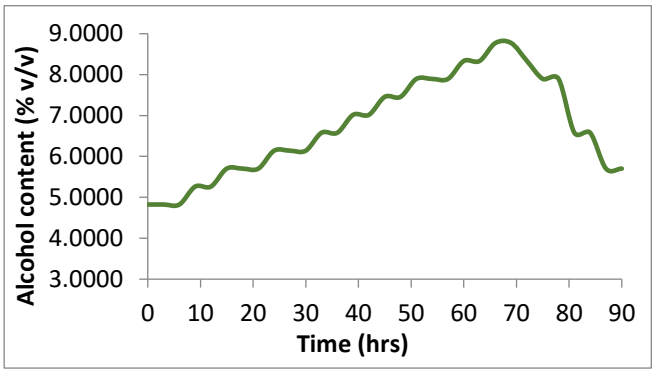


Figure 6. Yeast productivity through alcohol forming

IV. Conclusions

Of the two samples of nira that had been spread on NA and incubated for 24 hours, the nira tapped without APUS was denser and had a non-uniform size, from large to small, like powder. Upon further examination, there were three types of microorganism groups contained in the nira. AAB is a gram-negative bacteria that grows aerobically with rod-shaped cells, then LAB is a group of gram-positive bacteria. Under the microscope, the morphology of Saccharomyces species was observed to be round and ellipsoid. Again, the colonies of LAB and yeast were counted through the TPC method. Yeast had a lower colony count than LAB. This is because yeast requires three times longer to grow than LAB.

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