Isolation and characterization of acetic acid bacteria from palm sap (*Arenga pinnata* Merr.) for a starter culture in the production of Java plum (*Syzygium cumini* L.) vinegar

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Abstract. This study aims to isolate and characterize the AAB from the palm sap collected from Aceh Besar District, Aceh Province, Indonesia, in order to determine its viability as a starter culture in the production of Java plum vinegar. The palm sap was collected from three villages (Pagar Air, Seulimum and Lam Pakuk) after aging for 30 days. The samples were grown on Yeast-Glucose-Carbonate agar for AAB isolation and microbiological analysis and were examined for total cell counts, pH, total soluble solid (TSS), and alcohol content in triplicate. This study has successfully isolated 2 isolates from Pagar Air village (P1, P2), 1 isolate from Seulimum village (P3), and 2 isolates from Lam Pakuk village (P4, P5) with different colony characteristics. The characteristics of Gram negative, catalase positive, and oxidase negative suggest AAB were represented on P2 and P5 isolates only. Based on TSS analysis, the bacteria in palm sap from Pagar Air village could successfully convert sugar into alcohol. Therefore, P2 has the potential to be used as a starter culture in the java plum vinegar production process.

1. Introduction

Vinegar is a liquid produced from alcoholic and subsequent acinous fermentation from carbohydrates and it contains 3.75–5.0% acetic acid [1]. Vinegar is used in a variety of products from food flavorings and food preservatives, to healthy fermented beverages and home hygiene products. The nutrients and bioactive components in vinegar have been linked to a number of pharmacological effects, including antimicrobial effects, preventing cardiovascular diseases, cancer prevention, obesity prevention, antihypertensive, and decrease glucose level [2]. Due to its health benefits, vinegar is becoming increasingly utilized. Recently, vinegar has been made from rice [3], [4], olive oil mill wastewater [5], strawberries [6], sour cherries [7], lemon juice [8], citrus by-products [9], cereal [10], zalacca fruit [11], apples [12] and wine [13].

In Banda Aceh, Indonesia, java plum is one of potential fruits which can be processed to create vinegar because the sugar content reaches 14% (w/w). This plant is also rich in nutrients (vitamin A and C) and bioactive compounds such as terpenes, alkaloids, phenols, flavonoids, lipids [14], antioxidants [4, 5], antimicrobials, and antidiabetics [17]. The java plum tree is found in the subtropical Himalayas, India, Sri Lanka, Malaysia, western Indonesia, and Australia. It is a seasonal fruit that bears fruit only
once a year (around June-September). This fruit is classified as perishable, so it is rarely used as a fresh product. Currently, this fruit is used as a raw material for production in punch [18], pasta [19], and wine [20]. In the United States and European countries, java plum is considered a delicacy, and is marketed in a de-hydrated, seed-less form [16], [21]. In Indonesia, especially in Aceh, it is only consumed fresh or with added *pliek u* (grated-fermented-sun dried coconut meat).

There are two primary steps when producing vinegar, first fermentation involving yeasts as the first agent in alcoholic fermentation step, followed by acetic acid bacteria (AAB) in acetification step. One of the AAB sources is palm sap or palm juice which is obtained from the sap tree (*Arenga pinnata* Merr.). This study aims to isolate and characterize the acid-forming bacteria from palm sap in Aceh Besar which has the potential to be used as a starter culture in the production of java plum vinegar.

2. Materials and Methods

2.1. Materials

Fermented palm sap was collected from Pagar Air, Lam Pakuk, and Seulimum, three villages in Aceh Besar District, Aceh Province, Indonesia. The palm sap had been aged for 30 days. The samples were examined for bacteria isolation on the same day as collection.

2.2 Isolation and Characterization of Acetic Acid Bacteria

Acetic acid bacteria were isolated from Yeast extract- Glucose-Carbonat (YGC) which was incubated at 30°C for 48 hours in aerobe conditions and was re-streaked to attain pure isolates. The pure isolates were examined for colony and cell morphology and biochemical characteristics. The obtained isolates were identified using the guidelines in Bergey’s Determinative Bacteriology [22].

2.3 Chemical Characteristics and Total Cell Counts (TCC) of Palm Sap

The samples were examined for pH (ISTEK), total soluble solids (TSS; hand-held refractometer), and alcohol content (hydrometer alcohol) in triplicate. The inoculated media was incubated at 37°C for 2 days in aerobe conditions. The colonies grown on media GYC were counted ranging from 30 to 300 was used for further calculation according to the procedure set up by Gullo et al., [23]. The standard deviation for the data was then calculated.

3. Results and Discussions

3.1 Characterization of Isolates from Palm Sap

There were 5 AAB isolates in total which were 2 isolates from Pagar Air village (P1, P2), 1 isolate from Seulimum village (P3), and 2 isolates from Lam Pakuk village (P4, P5). The characteristic of AAB isolates can be seen in Table 1 and Figure 1. This assessment of the colony’s morphological characteristics is the preliminary step in identifying types of bacteria. This test cannot determine the type of bacteria intended, only help to describe, and classify the colonies based on the shape and other characteristics.

Based on Table 1, it can be seen that the colonies that grew were circular with flat edges. The colony color was typically milky white to gray (Figure 1). Further identification is carried out through observation under a microscope. Based on the microscopic observations, the results showed that all isolate cells had various shapes, from round to rods. In this test, the length of the cells ranged from 0.8 to 1.5 µm as in Figure 2. The isolates P1, P3, and P4 were Gram positive cells, while P2 and P5 isolates were Gram negative. The basic characteristics of AAB [24] are classified as Gram negative bacteria. This is determined by the red color seen on the bacterial cell wall as a result of the inability of the cell wall to retain the crystal violet color. Therefore, isolates P1, P3, and P4 are not classified as AAB.

The oxidase test results showed that P2, P3 and P5 samples showed negative oxidase, which was indicated by yellow strip oxidase (dimethyl-p-phenylenediamine oxalate). Oxidase-negative is one of the main characteristics of acetic acid bacteria. When acetic acid bacteria oxidize ethanol to acetic acid, it occurs in two consecutive catalytic reactions, first ethanol to acetaldehyde, then acetaldehyde is
immediately oxidized to acetate. If there is no aldehyde release, this indicates that the bacteria function changed from ethanol to acetic acid sequentially [24].

Table 1. Characteristics of acetic acid bacteria isolated from palm sap.

| Characteristics               | Isolates (Villages) |
|-------------------------------|---------------------|
|                               | P1 (Pagar Air)      | P2 (Pagar Air) | P3 (Seulimum) | P4 (Lam Pakuk) | P5 (Lampakuk) |
| Colony Morphology             |                     |                |              |                |               |
| Shape                         | Round               | Round          | Round        | Round          | Round         |
| Edge                          | Flat                | Flat           | Flat         | Flat           | Wavy          |
| Color                         | White - milk        | White - gray   | Gray         | Gray on the edges, milky white in the middle | White - gray  |
| Aspect                        | Burnish             | Blur           | Burnish      | Burnish        | Blur          |
| Elevation                     | Hilly, smooth       | Flat, rough    | Hilly, smooth| Hilly, smooth  | Flat, rough   |
| Cell Morphology               |                     |                |              |                |               |
| Gram stain                    | Positive            | Negative       | Positive     | Positive       | Negative      |
| Cell shape                    | Cocci-rods          | Rods           | Rods         | Cocci-rods     | Rods          |
| Cell length (µm)              | ± 0.8               | ± 1            | ± 1.5        | ± 1            | ± 1.2         |
| Oxidase Test                  | Positive            | Negative       | Negative     | Positive       | Negative      |
| Catalase Test                 | Positive            | Positive       | Positive     | Positive       | Positive      |

Figure 1. Colony and cell morphology (Olympus CX21; magnification 40X) of acetic acid bacteria isolated from palm sap in Pagar Air village (P1, P2), 1 isolate from Seulimum village (P3), and 2 isolates from Lam Pakuk village (P4, P5).

The estimation of AAB was increasingly tapered, marked by the catalase and oxidase tests. The results of catalase test showed that all types of isolates were catalase. This indicates that the five types of isolates were identified as capable of producing the enzyme catalase. The catalase enzyme functions to break H₂O₂ (hydrogen peroxide) into H₂O (water) and O₂ (oxygen). Hydrogen peroxide is a poisonous substance that kills bacterial growth. With the catalase enzyme, it means that bacterial growth is not disrupted, bacterial metabolism is able to run properly. Gomes et al., [24] explained that AAB are aerobic, Gram are negative, catalase are positive, and oxidase are negative, ellipsoidal microorganisms.
to stem-shaped cells that can appear independently, in pairs or chains. AAB species are known to have a high ability to oxidize alcohol, aldehyde, sugar, or sugar alcohol in the presence of oxygen.

3.2 Total Cell Counts of Palm Sap
The microorganisms in the Pagar Air sample showed the highest number of total cell count (25.43 Log CFU/ml) compared to the other 2 samples, as demonstrated in Figure 2. The results of Mussa's research [25] indicate microbial growth in palm sap grown on PDA and NA media for 15 hours yields 7.08 and 7.09 Log CFU/ml, respectively.

Bacterial growth is influenced by various factors, one of which is the availability of substrates in the growth medium. The high activity of microorganisms in palm sap Pagar Air samples after 30 days fermentation indicates that the water is rich in substrates necessary for microbe growth. Meanwhile, the other two samples had a lower number of microbes due to limited growth substrate.

![Figure 2](image_url)  
*Figure 2. Total cell counts of palm sap collected from Pagar Air, Seulimum, and Lam Pakuk villages.*

3.3 Chemical Characteristics of Palm Sap
Nutritional requirements may change with altered culture conditions like pH levels, amount of total soluble solid, and the concentration of ethanol. The pH results of palm sap from Pagar air, Seulimum, dan Lampakuk were 3.28±0.03, 3.35±0.05, and 3.31±0.04 respectively. The optimal pH for *Acetobacter aceti* [25] is about 4–6.3. Furthermore, Hommel [25] classified *Acetobacter* as acetophilic (optimum at pH 3.5), acetophobic (optimum at pH 6.5), and acetotolerant (optimum at both pH values). Generally, isolate is more resistant to acidic pH value when making vinegar.

The result of total soluble solid (TSS) of palm sap from Pagar air, Seulimum, dan Lampakuk were 9.5%±1.12, 10%±0.98, 11%±1.01 (w/w). While the ethanol concentration was 6.8%±0.09, 6.5%±0.06, 5.7%±0.23 (v/v). Ismail et al., [26] explained the sucrose content in fresh sap is about 15%. In the process of making vinegar, sugar is oxidized into alcohol by yeast, then the alcohol is used by acetic acid bacteria as the main substrate for vinegar. The alcoholic content when making vinegar [25] ranges from 10–15% (v/v ethanol). It is suspected that if there is a decrease amount of sugar during fermentation process for 30 days, then it takes even longer to convert all the sugar content into alcohol.
4. Conclusion

From the five isolates identified, only P2 and P5 were suspected to be acetic acid bacteria, indicated by the negative Gram, positive catalase, and negative oxidase characteristics. Further testing is needed in the molecular microbiology laboratory using genetic sequences to obtain more specific information.

The pH value of the three samples were at around 3.3, while the alcohol content of three samples were at around 6%. The lowest TSS was detected in Pagar Air palm sap (9.5%) indicating that the sugar content has converted into alcohol.

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