J-PEN Borneo: Journal of Agricultural Sciences
Volume VI, Number 7, October 2023
Pages: 57-64

E-ISSN: 2599-2872
P-ISSN: 2549-8150

ISOLATION PROCESS OF ACID-PRODUCING BACTERIA WITH MODIFIED MEDIA TO OBTAIN *ACETOBACTER ACETI* IN CASSAVA (*MANIHOT UTILISSIMA*) FERMENTATION

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ABSTRACT
Cassava (*Manihot utilissima*) is a type of high-carbohydrate plant that grows a lot in Indonesia. One of the most common and simple cassava processing is made into tape. One of the microorganisms in yeast is the genus Saccharomyces. Some species of this genus are considered very important in food production materials. One example is Saccharomyces cerevisiae, which is used in winemaking, bread, and beer. This study aims to: study the cassava fermentation process to obtain alcohol compounds as decomposition materials for acid-forming bacteria, knowing the characteristics of acid-forming bacteria in cassava tape fermentation, and getting isolates of Acetobacter aceti bacteria producing acetic acid in the cassava tape fermentation process. This research uses a qualitative descriptive method, with macroscopic, microscopic observations and gram painting on isolates of acid-forming bacteria that have been selected and successfully isolated from cassava fermentation. The results obtained by the colonies showed clear zones and did not show clear zones. Colonies that show clear zones are colonies that produce acid and react with carbonate ions; in the gram test obtained, a genus of bacteria obtained based on the results of isolation on cassava tape, namely some belonging to the genus Acetobacter with gram-negative (colored red) and some genus Lactobacillus with gram-positive (colored purple). The presence of a sour taste on the tape is caused by acid-forming bacteria after the presence of alcohol by the purples Saccharomyces cerevisiae.
Keywords: *Acetobacter aceti,* acetic acid, fermentation and *Saccharomyces cerevisiae*

INTRODUCTION

Fermentation is a chemical change in food materials caused by enzymes produced by microorganisms or those already present in the food itself. It is also a process of breaking down complex compounds into simpler ones involving microorganisms. Fermentation is one of the methods for processing and preserving food, extending its shelf life. One of the fermented food products is 'tape.' Proper fermentation is crucial in producing a high-quality final product [1]. Cassava (*Manihot utilissima*) is one of the carbohydrate-rich crops abundant in Indonesia. Cassava, with a high carbohydrate content of 63.6 grams and a low-fat content of 0.3 grams, can stimulate changes in blood glucose levels. This suggests that cassava has the potential to improve food security in Indonesia due to its high and healthy production [2].

One of the most common and straightforward ways to process cassava is by making 'tape.' The fermentation process improves the nutritional value of cassava. In addition to enhanced nutritional value, cassava tape has a softer texture and is easier to digest, facilitating better nutrient absorption. Cassava tape is obtained through the fermentation of cassava using fermentative microorganisms found in yeast [3].

Over time, there has been a shift in the dietary patterns of modern society towards the consumption of high-protein and high-fat foods with low fiber content, believed to be one of the triggers for various digestive system-related diseases. One approach to address this issue is by modifying the composition of gut bacteria, which can be achieved through the consumption of live bacteria maintaining a healthy balance of beneficial bacteria in the digestive system, referred to as probiotic bacteria. These probiotic bacteria are obtained from lactic acid bacteria known to be present

E-ISSN: 2599-2872
P-ISSN: 2549-8150

in cassava tape, which serves as a source for isolating lactic acid bacteria [4]. The objective of this study is to examine the characteristics of acid-producing bacteria, typically found in yeast, during the cassava tape fermentation process and to isolate Acetobacter aceti bacteria that produce acetic acid.

MATERIALS AND METHODS

Materials and Equipment

The materials used in the research included cassava, PCA (Plate Count Agar) media, NA (Nutrient Agar) media, $CaCO_3$ (calcium carbonate) analyzer, NaCl (sodium chloride), crystal violet Gram stain, iodine, distilled water (aquades), 70% alcohol, 96% alcohol, safranin, aluminum foil, plastic wrap, cotton, plastic, rubber, tissue, label paper, immersion oil. The equipment used included a Bunsen burner, glass beaker (Iwaki), inoculation loop, Erlenmeyer flask (Iwaki), analytical balance (Durascale), Heidolph MR82 magnetic stirrer hot plate, Sharp microwave, measuring glass (Iwaki), All American 75X autoclave, Memmert incubator, glass slides and cover slips (20x20 mm), Iwaki test tubes, test tube rack, Olympus microscope, Laminar Air Flow (LAF) HF-100 clean bench, Samsung refrigerator, micro pipette, pH meter, Anumbra Petri dishes, a mobile phone camera for documentation, and writing tools.

Methods

This research employed a qualitative descriptive research method, with macroscopic observations and biochemical testing of selected acid-producing bacterial isolates successfully isolated from cassava fermentation. Isolates of acid-producing bacteria were obtained from two different types of yeast:

a. Yeast code C, which is round and white, and suitable for the fermentation process in making 'tape'.
b. Yeast code N, also round and white, is commonly used by small-scale home industries to produce 'tape' in large quantities.

Procedure

Tape Production

The process of making cassava 'tape' involves several steps. Firstly, the materials and equipment are prepared. Cassava is cut into pieces, soaked for 12 hours, and steamed for 60 minutes. Afterward, the materials are drained for 1 hour. The yeast is then sprinkled evenly on the cassava and stored in containers wrapped in banana leaves, sealed tightly. The final step involves incubating the mixture for four days under anaerobic conditions [5].

Equipment Sterilization

All test tubes, Petri dishes, and Erlenmeyer flasks are soaked in sunlight and bleach for 24 hours. They are then thoroughly cleaned and dried. Petri dishes are wrapped in brown paper, while other equipment can be wrapped in aluminum foil. All equipment to be sterilized is placed in separate plastic bags and secured with rubber bands. Sterilization is done using an autoclave at 1 atm pressure and a temperature of 121°C for 15 minutes. Some equipment can be sterilized with 70% alcohol for those that cannot withstand high heat [6].

Medium Preparation

The isolation medium consists of PCA at 17.5 g/L and $CaCO_3$ at 10g/L. The ingredients are weighed and added to distilled water to make 1 liter. Sterilization is carried out using an autoclave

E-ISSN: 2599-2872
P-ISSN: 2549-8150

at 1 atm pressure and a temperature of 121°C for 15 minutes. Before solidifying, the medium is poured into sterilized Petri dishes under a Laminar Air Flow (LAF).

Sample Suspension

Cassava 'tape' samples are weighed (1g), placed in a dilution bottle, and diluted with sterile distilled water in test tubes to achieve dilutions from 10^{-1} to 10^{-6}. Sample suspension is performed within 24 hours of the sample being fresh.

Isolation and Purification of Microorganisms

The diluted samples are inoculated into the previously prepared medium, and colonies in the clear region are selected for isolation. A modified medium at pH 7.2 is used [7]. Sterilization is carried out using an autoclave at 1 atm pressure and a temperature of 121°C for 15 minutes, after which the medium appears turbid. A characteristic sign of acid formation is the presence of a clear zone around the colonies on the turbid medium.

Colonies with a clear zone are transferred with a sterile inoculation loop to a Nutrient Agar (NA) medium to cultivate the obtained bacteria. Subsequently, they are subcultured on slanted agar with Nutrient Agar (NA) medium and 50% NaCl.

Observation of Colony Morphology

The isolated bacteria are characterized by their growth on the culture medium. Observe colony growth on the culture medium and record the colony's growth pattern [8].

Gram Staining of Bacterial Isolates

The glass slide is cleaned with 70% alcohol. A circle is drawn on it to facilitate staining. One drop of sterile distilled water is added, followed by an inoculum of bacteria, which is spread evenly on the glass slide and fixed. Subsequently, crystal violet (Gram A) is dropped on it, left for 60 seconds, rinsed with sterile distilled water, and dried. Iodine solution (Gram B) is dropped on it, left for 60 seconds, rinsed with sterile distilled water, and dried. Then, the decolorizing solution, 96% alcohol (Gram C), is dropped on it, left for 30 seconds, rinsed with sterile distilled water, and dried. Finally, safranin (Gram D) is dropped on it, left for 60 seconds, rinsed with sterile distilled water, and dried. The glass slide is labeled to prevent mix-ups. It is then observed under a microscope at high magnification [9].

RESULTS AND DISCUSSION

Results of Acid-Producing Bacterial Isolates in the Cassava Tape Fermentation Process

Microbe Inoculation on $CaCO_3$-Modified PCA Media

The results of microbe isolation indicated that the microbial colonies obtained exhibited a clear zone around them. After spreading the sample on PCA media supplemented with $CaCO_3$ and incubating for 2-3 × 24 hours at 37°C, three culture colonies of acid-producing bacterial isolates were obtained. The results of acid-producing bacterial isolates can be observed in Table 1 and Figure 1.

Table 1. Results of Microbe Inoculation on $CaCO_3$-Modified PCA Media

No	Types of Yeast	Bacterial Code
1	Yeast C	C^{-1}
2	Yeast N	N^{-1} N^{-4}

J-PEN Borneo: Journal of Agricultural Sciences
Volume VI, Number 7, October 2023
Pages: 57-64

E-ISSN: 2599-2872
P-ISSN: 2549-8150

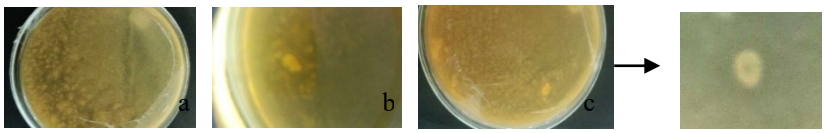

Figure 1. Acid bacteria colonies on $CaCO_3$-modified PCA (Plate Count Agar) media: (a). $^{C-1}$, (b). N-1, (c). N-4

Purification of Acid-Producing Microbes on NA Media

After obtaining the acetate acid bacterial isolates, it is necessary to cultivate the bacteria using the streak isolation method on NA media. Following incubation for 2-3 × 24 hours at 37°C, five culture colonies of acetic acid-producing bacterial isolates were obtained. The results of the purification of acid-forming microbes on NA media can be seen in Table 2 and Figure 2.

Table 2. Results of microbial isolates on NA media

No	Type of Yeast	Bacterial Code
1	Yeast C	C^{-11}
		C^{-12}
		C^{-13}
2	Yeast N	N^{-1}
		N^{-4}

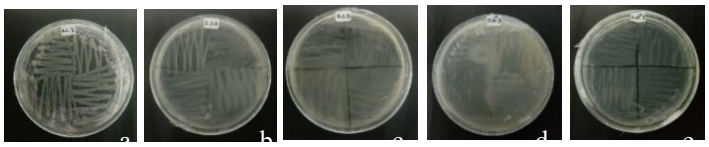

Figure 2. Acid bacterial colonies on NA media (Nutrient Agar) (a). C^{-11},(b). C^{-12},(c). C^{-13},(d). N^{-1}, (e). N-4

Microbial Culture

Bacteria were cultured on NA media modified with 50% NaCl using an agar slant method to increase the bacteria yield. After incubation, 15 culture colonies of acetic acid-producing bacterial isolates were obtained. The results of the acetic acid-producing bacterial culture can be seen in Table 3.

Table 3. Results of bacterial culture on plant agar

No	Type of Yeast	Bacterial Code
1	Yeast C	$C^{-1}a$
		$C^{-1}b$
		$C^{-1}c$
		$C^{-11}a$
		$C^{-11}b$
		$C^{-11}c$
		$C^{-12}a$
		$C^{-12}b$
		$C^{-12}c$
		$C^{-2}a$
		$C^{-2}b$
		$C^{-2}c$
2	Yeast N	$N^{-43}a$
		$N^{-43}b$
		$N^{-43}c$

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Preparation of Colonies for Gram Stain Test of Isolate Cells

In the Gram stain test, the microbial cells are classified as either Gram-negative or Gram-positive bacteria. The observation results indicate that some isolates have the same cell shape, which is rod-shaped, and they exhibit both Gram-negative and Gram-positive characteristics. Purple color indicates Gram-positive bacteria, while red color indicates Gram-negative bacteria. The results of the Cytology Test with the Gram technique can be seen in Table 4 and Figure 3.

Table 4. Results of Cytology Test with Gram Technique

Bacterial Code	Color	Microscopic Shape	Explanation
$C^{-1}a$	Red	Rod	Negative
$C^{-1}b$	Red	Rod	Negative
$C^{-1}c$	Purple	Rod	Positive
$C^{-1}1a$	Purple	Rod	Positive
$C^{-1}1b$	Red	Rod	Negative
$C^{-1}1c$	Red	Rod	Negative
$C^{-1}2a$	Red	Rod	Negative
$C^{-1}2b$	Red	Rod	Negative
$C^{-1}2c$	Red	Rod	Negative
$C^{-2}a$	Purple	Rod	Positive
$C^{-2}b$	Purple	Rod	Positive
$C^{-2}c$	Purple	Rod	Positive
$N^{-4}3a$	Red	Rod	Negative
$N^{-4}3b$	Red	Rod	Negative
$N^{-4}3c$	Purple	Rod	Positive

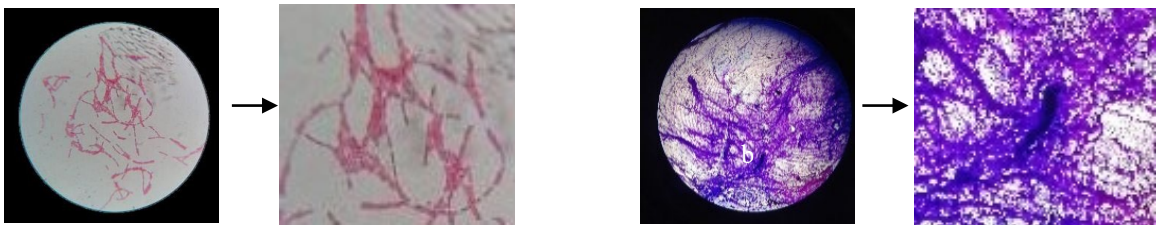

Figure 3. Results of Cytology Test with Gram Technique (a) Gram-negative and (b) Gram-positive

Discussion

Inoculation of Microbes on $CaCO_3$-Modified PCA Media

When inoculating cassava tape samples on $CaCO_3$-modified PCA media, a cloudy medium with clear zones around the colonies is obtained. Colonies showing clear zones are those that produce acid. The acid secreted from the colonies will react with the $CaCO_3$ in the PCA media, resulting in a clear zone around the colony. These colonies are predicted to be acid bacteria [10].

Before using the media, it is essential to check the pH and sterility of the growth medium. Most pathogenic microorganisms grow optimally at pH 6.5 – 7.5, and generally, bacteria do not grow at extremely acidic or alkaline pH levels. PCA media has an optimal pH of 7.0±0.2 at a final temperature of ±25oC, making it suitable for bacterial growth [11].

Purification of Acid-Forming Microbes on NA Media

The ability of isolates to grow at various pH levels is tested by inoculating selected isolates on NA media (Nutrient Agar). The pH levels of NA media are adjusted to 6, 7, 8, and 9. The media is then sterilized in an autoclave at 121°C and 2 atm pressure for 15 minutes. After cooling, the media is poured into Petri dishes aseptically and prepared as a bacterial growth medium [12].

E-ISSN: 2599-2872
P-ISSN: 2549-8150

Microbial Culture

The addition of salt concentration does not affect the activity of microorganisms in producing acid. In the fermentation of *Acetobacter aceti*, it can grow well under 10% NaCl concentration. However, fermentation conditions with a 5% NaCl concentration tend to inhibit the activity of microorganisms. The use of these microorganisms tends to produce acid, namely *Acetobacter aceti*. The metabolism of this bacterium results in the production of acetic acid. Adding salt in fermentation can inhibit the growth of Gram-negative microorganisms, while in this study, *Acetobacter aceti* is a Gram-negative bacterium [13]. This inhibition is because the cell walls of Gram-negative bacteria are more susceptible to plasmolysis than Gram-positive bacteria. The cell wall of Gram-negative bacteria has a thinner structure with 10% peptidoglycan and an outer membrane composed of lipoproteins, polysaccharides, and phospholipids. The inhibition of *Acetobacter aceti*'s activity by 5% NaCl concentration is also shown in the oxalate content in the biomass.

Fermentation Process for Obtaining Acid Bacteria from Cassava

Cassava is a complex carbohydrate composed of two monosaccharide bonds, referred to as polysaccharides. Important polysaccharides include starch, dextrin, glycogen, and non-starch polysaccharides. Starch is the main carbohydrate reserve consumed by humans worldwide and is found in grains, tubers, and seeds [14].

The preparation of cassava tape involves several stages. Initially, the equipment and materials are prepared, and cassava is cut into pieces. The cassava is then soaked for 12 hours and steamed for 60 minutes. After that, the material is drained for 1 hour. Subsequently, yeast is sprinkled evenly on the cassava and stored in a container wrapped in banana leaves and tightly sealed. The final step involves four-day incubation under anaerobic conditions [15].

The fermentation process of cassava tape can be broken down into four stages: starch is converted into sugar, sugar into alcohol, alcohol into organic acid, and organic acid into ester [16]. During the fermentation process, various compounds, including acetic acid, are produced due to the activity of acid-producing bacteria.

The ester compounds produced during the fermentation process contribute to the alcoholic, less alcoholic, or slightly alcoholic aroma of cassava tape. This is attributed to the presence of carbonyl compounds, acids, and other substances like ethyl benzene and propyl benzene [17].

In this study, the isolation method used is the dilution method, where cassava tape is diluted in various steps, ranging from 10^{-1} to 10^{-6} dilutions. Different dilutions are prepared to obtain varying concentrations and reduce contamination levels, making it easier to observe the growth of bacteria or colonies.

Acetic acid is produced through fermentation. The ongoing fermentation process transforms sucrose present in the fermentation solution into alcohol, which then further converts into acetic acid [18]. Acetic acid is an organic compound with a colorless appearance, a pungent odor, a sharp acidic taste, and solubility in water, alcohol, glycerol, and ether.

Preparation of Colonies for Isolation of Gram Stain Test

In the macroscopic examination, acid bacteria isolates with different characteristics are observed by examining colony shape, elevation, and margin. The morphology of colonies on mixed media exhibits various surface properties, such as size (small or large), round shape, elongated shape, even or uneven margins, surface smoothness or roughness, surface gloss, matte surface, and

E-ISSN: 2599-2872
P-ISSN: 2549-8150

various colors (white, yellowish, etc.) [19]. Colony shapes can range from intact to wavy and serrated.

In the Gram stain test, the goal is to determine the Gram characteristics of the observed bacteria. The results of the Gram type of bacteria are presented in Table 4. The staining process starts with crystal violet, resulting in a purple color. Then, iodine solution is used as a mordant, maintaining the purple color. The third step involves decolorization with 95% ethanol. In the third step, Gram-positive bacteria will remain purple, while Gram-negative bacteria will lose color. The final step is staining with safranin, where Gram-positive bacteria will appear light purple, and Gram-negative bacteria will appear red [19[. The staining process can be categorized into two types: simple staining or complex staining, such as the Gram stain, which reveals the distinction between Gram-positive and Gram-negative bacteria based on differences in cell wall structure.

Acetobacter aceti bacteria can convert ethanol into acetic acid through oxidation, and they are classified as Gram-negative bacteria, indicated by their red coloration. *Acetobacter aceti* belongs to the Pseudomonadaceae family and has characteristics that include short rod-shaped or spherical cells, Gram-negative bacteria, motile and non-motile cells, lack of endospores, non-pathogenicity, aerobic nature, energy derived from the oxidation of ethanol to acetic acid, and the ability to thrive in water, solids, leaves, fruits, and more. *Acetobacter aceti* is classified as a peroxide-producing bacterium if it accumulates acetate. Lactobacillus bacteria are Gram-positive and are identified by the purple coloration of their cells. This is due to the acidic nature of these bacteria. Gram-positive bacteria have a thick peptidoglycan cell wall, so they retain the purple color when stained with crystal violet [20].

CONCLUSION

1. Cassava is a complex carbohydrate. The fermentation process of cassava tape involves four stages: starch to sugar, sugar to alcohol, alcohol to organic acid, and organic acid to ester. The ester compounds produced during the fermentation process contribute to the alcoholic, less alcoholic, or slightly alcoholic aroma of cassava tape.
2. Based on the results of isolating cassava tape (*Manihot utilissima*), some of the bacterial isolates belong to the Acetobacter genus (C^{-1}a, C^{-1}b, C^{-1}1b, C^{-1}1c, C^{-1}2a, C^{-1}2b, C^{-1}2c, N^{-4}3a dan N^{-2}3b). They exhibit characteristics such as round white colonies, ellipsoidal cell shapes, rod-shaped cells, single or chained cells, and are Gram-negative (red coloration). Some isolated samples belong to Gram-positive bacteria (C^{-1}c, C^{-1}1a, C^{-2}a, C^{-2}b, C^{-2}c dan N^{-4}3c), indicated by purple cell coloration with similar rod-shaped (bacilli) cell morphology, and they belong to the Lactobacillus genus.
3. Some colonies exhibit clear zones, while others do not. Colonies with clear zones are those that produce acid.

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