JPPIPA 9(2) (2023)

Jurnal Penelitian Pendidikan IPA

Journal of Research in Science Education

http://jppipa.unram.ac.id/index.php/jppipa/index

Isolation and Characterization of Indigenous Lactic Acid Bacteria from *Pakatikng Rape*, Dayak's Traditional Fermented Food

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Received: December 25, 2022
Revised: February 19, 2023
Accepted: February 25, 2023
Published: February 28, 2023

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DOI: 10.29303/jppipa.v9i2.2801

Abstract: West Kalimantan is one of the provinces with a variety of traditional foods. One of them is pakatikng rape, a traditional Dayak fermented food made from kelampai fruit. The fermentation process involves lactic acid bacteria which act as food preservatives because they have inhibitory substances known as bacteriocins. Exploration of lactic acid bacteria in the local food fermentation process, especially in West Kalimantan, has not been widely carried out. Therefore, the purpose of this research is to obtain LAB from kelampai fruit fermentation. The research methods included fermentation of kelampai fruit, isolation of LAB, screening for acid-producing LAB, characterization of colony morphology, gram staining, endospore staining, motility test, catalase test, and antibiotic sensitivity test. The results of the study obtained nine isolates that are capable of producing acid and have general characteristics of lactic acid bacteria, namely round cell shape, gram-positive, non-endospores, non-motile and catalase negative and four isolates that are resistant to antibiotics, namely KL-10 resistant to erythromycin, KL-15 and KL-19 resistant to Ofloxacin, and KL-20 resistant to amoxicillin.

Keywords: Fermentation; Lactic Acid Bacteria; Traditional food

Introduction

West Kalimantan is one of the provinces with abundant traditional food wealth. One of them is Pakatikng Rape or Jaruk Rape, which is a fermented traditional food typical of the Dayak tribe. Pakating Rape is made with the basic ingredients of kelampi fruit or also known as tapos fruit (Elateriospernum tapos). The Dayak people only consume fermented kelampai fruit. This is done because fresh kelampai fruit contains cyanide which can cause poisoning when consumed directly (Ngamriabsakul & Kommen, 2009; Husin et al., 2013).

Traditionally fermented food, in the manufacturing process occurs spontaneously by utilizing natural microbes from the environment (Anggraini et al., 2019). One of the microbes that play an important role in fermentation is lactic acid bacteria (Novelia et al., 2020; Leisner et al., 2002; Chuah et al., 2016). Lactic acid bacteria have an important role in providing food preservation effect by inhibiting microbial (pathogen) decay. This makes lactic acid bacteria classified as a food-grade microorganism category (Sofiana et al., 2020).

The ability of lactic acid bacteria to preserve food because lactic acid bacteria have substances with the ability to inhibit the growth of spoilage microbes (pathogens) (Sofiana et al., 2020) known as bacteriocins (Alvarez-Sieiro et al., 2016). Bacteriocins have been studied extensively, and several have been developed commercially for their ability to preserve food and to exhibit therapeutic antimicrobial activity. Many bacteriocins are thermostable and active over a wide pH range. In addition, most bacteriocins are non-immunogenic, and are generally colorless, odorless, and tasteless. These characteristics make bacteriocins very attractive for food preservation and healthcare

How to Cite:
Rahayu, H.M., & Setiadi, A. E. (2023). Isolation and Characterization of Indigenous Lactic Acid Bacteria from Pakatikng Rape, Dayak's Traditional Fermented Food. *Jurnal Penelitian Pendidikan IPA*, *9*(2), 920–925. https://doi.org/10.29303/jppipa.v9i2.2801

applications (Alvarez-Sieiro et al., 2016).

Seeing the great potential of lactic acid bacteria and the availability of lactic acid bacteria in the market which is still very limited and the utilization of lactic acid bacteria in local fermented foods has not been done much, therefore efforts to explore the potential of lactic acid bacteria, especially those originating from local West Kalimantan products such as Fermentation of kelampai fruit is important to do.

Method

Manufacture of kelampai fruit fermentation

The fruit of the kelampai is first washed thoroughly and then boiled for 1-2 hours. After that the boiled kelampai fruit is soaked overnight, then put in a jar or crock and incubated for one month to produce kelampai fruit fermentation.

Isolation of lactic acid bacteria from kelampai fruit fermentation

One gram of sample was added to 9 ml of sterile distilled water, homogenized using a vortex (a 10-1 dilution). Serial dilutions were made up to 10-6 dilutions. From dilution 10-6 isolated using MRS agar media in a petri dish with the spread plate method. The culture was incubated at 30 ^{o}C for 48 hours. The culture was purified on MRS agar and incubated at 30 ^{o}C for 48 hours. Pure cultures were stored on MRS media so that it was slanted, at 4-10 ^{o}C.

Screening for acid-producing lactic acid bacteria

To select lactic acid bacteria that have the potential to produce acid, a selection was made using MRSA+ $CaCO_3$ 0.5% media. Incubation was carried out for 48 hours at 26°C. The presence of a clear zone around a bacterial colony indicates that the bacteria produce acid. Measurement of the size of the clear zone is carried out to determine the ability of bacteria to produce acid with the clear zone index formula with the equation:

$$\text{Acid clear zone index} = \frac{\text{diameter of the acid clear zone}}{\text{colony diameter}} \quad (1)$$

Characterization of lactic acid bacteria

The isolates obtained were then characterized through testing; gram stain, endospore stain, catalase assay, motility.

Gram stain

The smear preparations were made in glass objects, fixed over a Bunsen flame. The preparation is dripped with a solution of purple crystals, left for 60 seconds and washed with running water and dried. The preparation is dripped with iodine solution and left for 2 minutes, washed with running water and dried. The preparation is dripped with 96% alcohol until the purple color disappears. The preparations were dripped with safranin and left for 30 seconds, washed with running water and dried. The preparation is dripped with immersion oil. The preparations were observed under a microscope, the gram test was positive if the cells were purple and negative if the cells were red.

Endospore staining

The preparations for the preparations were made in glass objects, fixed over a Bunsen flame. The preparations were covered with straw paper and dripped with green malachite, cooled. The preparation is placed on a wire heated over boiling water for 5 minutes. The preparations were washed carefully with running water. The preparation was dripped using safranin, left for 60 seconds, then washed with running water and dried carefully. The preparations were observed under a microscope, the test was positive if the vegetative cells were red and the spores were green.

Catalase test

Isolate from agar slanting is taken in one loop, then smeared on a glass object that has been given alcohol. The object glass is dripped with 3% H_2O_2 solution. The formation of gas bubbles was observed in the preparations. If there are gas bubbles, the catalase test is positive.

Motility test

Isolates from oblique agar were poked onto semi-solid upright agar and then incubated for 48 hours at 30oC. Bacterial motility test was observed. The motility test is positive if colony growth is widespread on the agar.

Antibiotic sensitivity test was carried out using the disc diffusion method

One ose of lactic acid bacteria per isolate, cultured with 10 ml of MRS Broth using a test tube and incubated at 37°C for 24 hours. After completion of incubation, the lactic acid bacteria isolate was smeared evenly using sterile cotton onto the surface of MRS Agar in a petri dish. The antibiotic discs used consisted of three types of antibiotics, each 6 mm in diameter, namely erythromycin (E), amoxicillin (AML), and ofloxacin (OFX). The three types of discs were placed on the surface of the MRS Agar media which had been smeared with lactic acid bacteria using tweezers. Then incubated at 37°C for 48 hours. After completion of incubation, the inhibition zone on the growth of lactic acid bacteria was measured with a caliper.

Result and Discussion

Fermentation is one of the oldest food preservation techniques in the world (Anggraini et al., 2019). The kelampi fermentation process is a spontaneous fermentation without the addition of salt with a fermentation time of one month and is carried out on a small scale through a traditional fermentation process. Fermented kelampai seeds have a dark brown color and a strong odor (Husin et al., 2013) (Figure 1b).

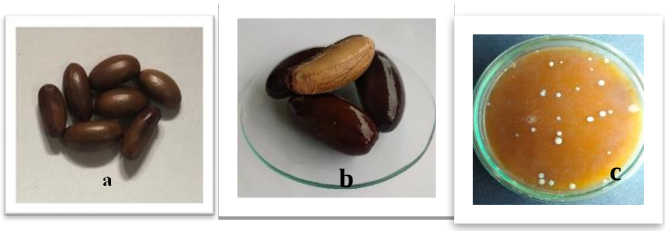

Figure 1. (a) Fresh Kelampai Fruit; (b) Coconut Fermentation; (c) Kelampai lactic acid bacteria isolate

Fermented kelampai has a better nutritional content than fresh kelampai (Muhamad et al., 2021). The fermentation process can also extend the shelf life because fermented lampai has a lower moisture content than fresh lampai. Low humidity levels can inhibit microbial growth (Muhamad et al., 2021). The kelampai fermentation process also reduces heavy metal content, increases potassium content, increases fat content and reduces cyanide content (Husin et al., 2013; Muhamad et al., 2021). Fermentation in general can also improve the taste (Anggraini et al., 2019).

Screening of acid-producing lactic acid bacteria isolates

The results of the isolation of kelampai lactic acid bacteria obtained a total of 24 isolates with nine isolates of lactic acid bacteria that had a clear zone around the colony ≥ 4 mm (Table 1). The clear zone that forms around the colony is the result of the reaction between lactic acid produced by lactic acid bacteria and calcium carbonate ($CaCO_3$) added to the MRSA medium. (Anggraini et al., 2019). The addition of $CaCO_3$ into the growth medium is an early confirmation of lactic acid bacteria. Lactic acid bacteria will use glucose in the media as an energy source and produce secondary metabolites in the form of acid which is visible as a clear zone around the colony (Nuryady et al., 2014).

Table 1. Diameter of the acid clear zone of lactic acid bacteria isolates

Isolate Code	Acid Clear Zone Diameter (Mm)
KL-10	5
KL-12	7.75
KL-13	5.75
KL-14	4
KL-15	4
KL-16	8
KL-18	7.75
KL-19	6.75
KL-20	9

Characterization of lactic acid bacteria colonies

Characterization of lactic acid bacteria colonies was carried out on nine isolates by observing the shape, color, edges, elevation, and surface of the lactic acid bacteria colonies. The observations showed that the nine isolates had the same characteristics, namely having a cream color, circular shape, entire edge, convex elevation with a smooth surface (Table 2).

In general, lactic acid bacteria colonies have general characteristics, namely circular in shape (Nasution et al., 2020), white to yellowish in color, entire margin, convex elevation and smooth surface (Siregar et al., 2014; Manzoor et al., 2016; Mahulette et al., 2018; Rahayu & Qurbaniah, 2019; Nasution et al., 2020; Nasri et al., 2021).

Table 2. Colony morphology characteristics of lactic acid bacteria isolate from kelampai fermentation

Characterization of Colony Morphology	KL-10	KL-12	KL-13	KL-14	KL-15	KL-16	KL-18	KL-19	KL-20
Color	cream	cream	cream	cream	cream	cream	cream	cream	cream
Form	circular	circular	circular	circular	circular	circular	circular	circular	circular
Edge	entire	entire	entire	entire	entire	entire	entire	entire	entire
Elevation	convex	convex	convex	convex	convex	convex	convex	convex	convex
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth

Gram stain

Morphological characterization of isolate cells was carried out through a gram staining process. Gram staining is a fundamental characterization for determining the phenotypic bacteria (Smith & Hussey, 2005). The gram staining process uses crystal violet as the main dye, iodine as a mordant, alcohol aetone as a decolorizing agent and safranin as a counterstaining agent (Bartholomew & Mittwer, 1883; Smith & Hussey, 2005). Gram staining is used to differentiate microbes

based on cell ultrastructure, especially the type of cell wall (Toole, 2016; Smith & Hussey, 2005)

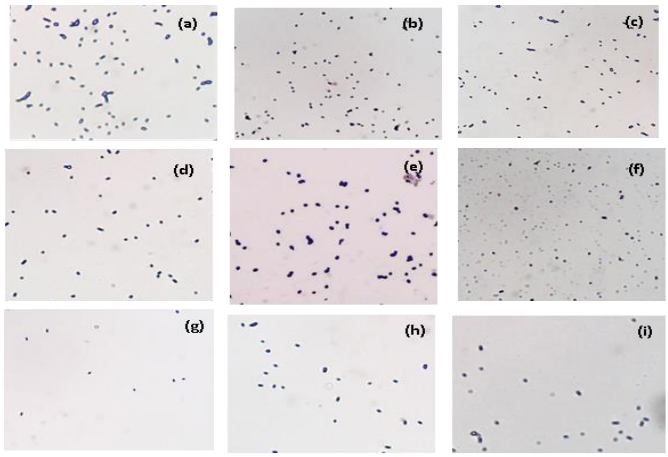

Figure 2. Gram staining results of isolates (a) KL-10; (b) KL-12; (c) KL-13; (d) KL-14; (e) KL-15; (f) KL-16; (g) KL-18; (h) KL-19; (i) KL-20

The results of gram staining (Figure 2) showed that all isolates of lactic acid bacteria had purple cells with coccus cell shapes. The purple color indicates that the bacterial cells are gram-positive bacteria. The formation of a purple color is because the bacterial cell has a thick peptidoglycan layer so that it can help maintain the crystal violet color during the decolorization process. (Toole, 2016). Gram positive character is a common characteristic of lactic acid bacteria (Yanti & Dali, 2013; Nurin et al., 2017; Yusmarini et al., 2017; Fallo et al., 2021; Kurniati et al., 2021).

Endospore staining results

Endospore staining was one of the early techniques used for the characterization of bacteria (Hussey & Zayaitz, 2007).

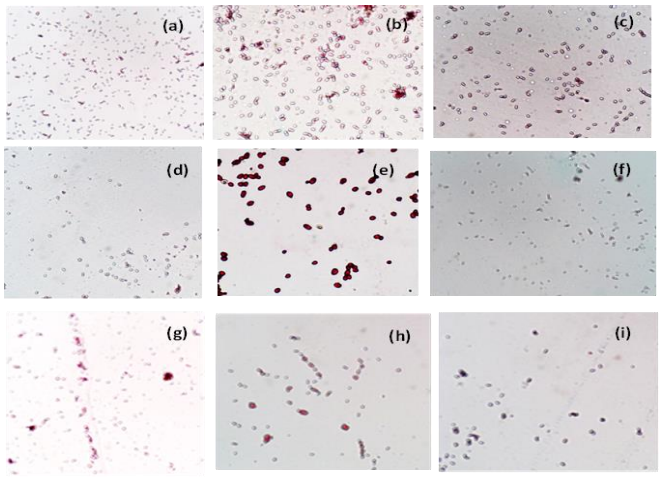

Figure 3. Endospore staining results of lactic acid bacteria isolate Kelampai (a) KL-10; (b) KL-12; KL-13, (d) KL-14; (e) KL-15; (f) KL-16; (g) KL-18; (h) KL-19; (i) KL-20

Endospores have different characteristics from vegetative cells so that this staining can be used to differentiate between spore and nonspore bacteria. Endospores have structures that are resistant to heat, radiation, chemicals, and other agents that generally cause death in bacteria. The results of endospore staining using green malachite showed that all isolates were non-endospore because the cells were only stained red (Figure 3). This is in line with previous research that one of the general characteristics of lactic acid bacteria is non-endospore (Yanti & Dali, 2013; Nurin et al., 2017; Yusmarini et al., 2017; Fallo et al., 2021).

Motility test

The physiological characterization carried out was the motility test. The motility test showed that all lactic acid bacteria isolates were non-motile. This can be seen from the distribution of lactic acid bacteria colonies grown on semi-solid MRS media which did not spread because the colonies did not have flagella. Non-motile nature is also a general characteristic of lactic acid bacteria (Fallo et al., 2021). The next characterization of lactic acid bacteria includes the catalase test. The test results showed that KL-15 showed positive catalase results while KL-10, KL-12, KL-13, KL-14, KL-16, KL-18, KL-19, KL-20 showed negative catalase (Table 3).

Table 3. Motility and catalase test results of isolates of kelampai lactic acid bacteria

Isolate Code	Motility Properties	Catalase Test
KL-10	Non Motil	Negative
KL-12	Non Motil	Negative
KL-13	Non Motil	Negative
KL-14	Non Motil	Negative
KL-15	Non Motil	Positive
KL-16	Non Motil	Negative
KL-18	Non Motil	Negative
KL-19	Non Motil	Negative
KL-20	Non Motil	Negative

The catalase test is performed to detect the presence of the catalase enzyme in bacteria. A negative test result is indicated by the absence of bubbles when the isolate is dripped with H_2O_2. This indicates that bacteria do not have the catalase enzyme which will convert hydrogen peroxide (H2O2) into water and oxygen ($2H_2O$+ catalase enzyme ($2H_2O+O_2$). The results of this test are in line with his research.

Sensitivity test of lactic acid bacteria to antibiotics

The sensitivity test of lactic acid bacteria to antibiotics was carried out on three types of antibiotics namely Amoxicillin (AMX), Ofloxacin (OFX), and Erythromycin (E) (Table 4).

Table 4. Antibiotic Sensitivity Test Results

Isolate Code	Obstacles zone (mm)		
	Amoxicillin (AMX)	Ofloxacin (OFX)	Erythromycin (E)
KL-10	7.5	7	0
KL-12	10.75	7.75	7.5
KL-13	6.75	7.75	10.25
KL-14	8	6.75	8.5
KL-15	6	0	6
KL-16	6.75	7.75	9
KL-18	8.5	9.5	8.5
KL-19	9.25	0	7.25
KL-20	0	6.5	7

The results of the antibiotic sensitivity test showed that isolate KL-10 was resistant to erythromycin, KL-15 and KL-19 were resistant to ofloxacin, and KL-20 was resistant to amoxicillin. Antibiotic resistance in bacteria can occur through the process of transferring genes in plasmids, one of which is through conjugation (Monika et al., 2017). The sensitivity test of lactic acid bacteria to antibiotics is important for which resistance genes are generally present in plasmids.

Conclusion

Based on the results of the study it can be concluded that the nine isolates of lactic acid bacteria obtained were able to produce acid and had general characteristics of lactic acid bacteria, namely round cell shape, gram positive, non-endosporeal, non-motile and catalase negative and four isolates that were resistant to antibiotics namely KL-10 resistant to erythromycin, KL-15 and KL-19 resistant to ofloxacin, and KL-20 resistant to amoxicillin.

Acknowledgements

Researchers would like to thank LPPM Muhammadiyah University Pontianak for the financial support provided.

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