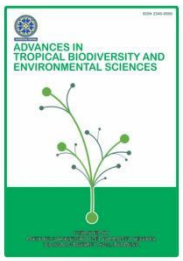

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Exopolysaccharides-Producing Lactic Acid Bacteria in Marinated Pakoba (*Syzygium luzonense* Merr.) Fruit

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Abstract. Pakoba fruit is one of the endemic flora of North Sulawesi which is rich in nutrients and has the potential to be fermented. The fermentation process occurs due to the bacteria in the fruit, including lactic acid bacteria (LAB). One of the interesting products from LAB fermentation is that it can produce secondary metabolites which have the potential to produce exopolysaccharides, namely high molecular weight and biodegradable polymers. This research aims to determine whether LAB can be obtained from fermentation of Pakoba marination and what types of LAB produce exopolysaccharides based on their phenotypic characteristics. This research uses a qualitative descriptive method to characterize the type of LAB and observe the exopolysaccharides produced by the bacteria. This research succeeded in isolating and identifying 10 LAB isolates based on their morphological and biochemical characters. Four isolates named MP(1)5.2, MP(1)7.1, MP(3)6.3, MP(3)7.2 can produce exopolysaccharides with a soft character but not mucus, where bacterial exopolysaccharides are believed to increase probiotics in the digestive tract. Genus level recognition (generic assignment) based on profile matching revealed that these four bacteria were from the genus *Lactobacillus*.

Keywords: exopolysaccharide, lactic acid bacteria, marinated of Pakoba fruit

I. INTRODUCTION

Pakoba fruit is one of the endemic flora of North Sulawesi which is rich in nutrients, has a sour taste and fermentable because of its high water and natural sugar content, as well as the bioactive compounds as the substrate for bacteria. This fruit contains saponins which have potential hypocholesterolemic and immunomodulatory effects [1], and contains flavonoids which can be used as antioxidants, believed to be one of the secondary metabolite compounds that are beneficial for humans [2]. This fruit is widely consumed by the local society by fermenting it into a wet fruit marinade or locally known as *rujak Pakoba*.

Fermentation occurs due to the activity of bacteria contained in the fruit. Bacteria play an important role in various biochemical processes, especially in the fermentation of food and beverage products [3].

Bacteria can be classified into several types, including lactic acid bacteria which are famous for their ability to produce lactic acid from carbohydrates through fermentation. Fruit, vegetables, meat, and fermented foods often become natural habitats for lactic acid bacteria [4]. Lactic acid bacteria are classified as microorganisms that are safe to use in food because they do not produce toxins and non-pathogenic. Therefore, they are included in the General Recognized As Safe (GRAS) category and are beneficial for health [5]. Fermentation of lactic acid bacteria can produce interesting products that have the potential to produce exopolysaccharides via secondary metabolites [6].

Exopolysaccharides are extracellular macromolecules that are synthesized and released by bacteria in the form of capsules or mucus layers [7]. The formation of extracellular polysaccharide (EPS) polymers can also occur due to the catalytic role of the fructosyltransferase (FTF) enzyme originating from LAB. Fructosyltransferase (FTF) is an enzyme that catalyzes the formation of fructose-fructose bonds which is the main component of EPS. The FTF gene functions as a template to produce FTF, the FTF enzyme then plays a role in the synthesis of fructan which is part of EPS. The benefits of exopolysaccharides include prebiotics, emulsifiers, stabilizers and water binders which also have the ability to form gels in food products [8]. Exopolysaccharides can improve health by stimulating the

immune system, inhibiting tumor growth, and increasing the ability of macrophages and lymphocytes to strengthen the immune system [4].

This research focuses on the process of isolation and identification of exopolysaccharide-producing lactic acid bacteria obtained from marinating Pakoba (*Syzygium luzonense* Merr.) using solid media exopolysaccharide test technique. Qualitative methods including morphological observation and biochemical analysis, as well as identification of lactic acid bacteria at the genus level (generic assignment) based on profile matching were performed.

II. METHODS

Location, Tools, and Materials

This research was carried out in the Microbiology Lab, Department of Biology, FMIPA-K, Universitas Negeri Manado, from April to June 2025. Equipments used included knives, tweezers, petri dishes, stir sticks, erlenmayer, glass jars, test tubes, tubes, blue tips, aluminum foil, wrappers, cotton, sterile plastic and rubber, autoclaves, laminar air flow, incubators, ose, bunsen burner, analytical balance, scales, hot plates, light microscopes, microscope slide, graduated cylinders and beakers. The research materials used were Pakoba (*Syzygium luzonense* Merr.), water, sucrose, spirit fluid, waterone, Man Ragosa Sharpe Broth (MRSB), agar, 70% $CaCO_3$, 3% $CaCO_3$, malachite green solution, safranin solution, crystal violet solution, iodine (lugol) solution, 96% alcohol, 95% alcohol.

Research methods

This research employed a qualitative descriptive approach. The stages carried out begin by taking samples of Pakoba fruit directly from the tree, followed by making fermented Pakoba fruit marinade, then isolating bacteria from the marinated Pakoba fruit and identifying them based on morphology and biochemistry.

Research Procedures

Sample Preparation

This research used samples of ripe Pakoba fruit (*Syzygium luzonense* Merr.) taken from Rinegetan Village, West Tondano District, Minahasa Regency by picking them directly from the tree, then formulating them into a wet Pakoba fruit marinade, cooking and cooling them and putting them in a jar as a fermenter. The glassware used is washed clean, dried, covered with aluminum foil and then wrapped in heat-resistant plastic. Then the glassware was sterilized using an autoclave at 121℃ for 20 minutes [9].

Pakoba Fruit Fermentation

The Pakoba fruit that has been previously cut is then soaked in a whiting solution for 30 minutes, then washed thoroughly. Subsequently, 300 grams of granulated sugar was added into boiling water, then continue by adding 1 piece of cinnamon, 2 tablespoons of vinegar, 0.25 teaspoon of salt, and a few drops of yellow food coloring into the water. The fruits were mixed with this after the sugar has dissolved and were cooked until the sugar is absorbed. These were then put into a closed jar as a fermentation container for 3 days. Isolation of lactic acid bacteria was performed on the first and third days of fermentation in MRS media using the spread plate method [10].

Medium Preparation

MRSA media was made using 250 mL of distilled water, 13.8 grams of MRS media, 5 grams of agar and 2.5 grams of $CaCO_3$ 3. These were put into an erlenmyer and heated until reaching the boiling point. Sterilization was then carried out for ¼ hour using an autoclave at a temperature of 121℃. MRSB media was made by measuring 150 mL of distilled water and weighing 7.83 grams of MRSB media into an erlenmayer and heated until boiled on a hot plate and then covered with cotton, followed by a sterilization using an autoclave at 121℃ for ¼ hour [4].

Isolation and Incubation of Bacteria

1 mL of Pakoba marinade was taken aseptically and added to 9 mL of diluent solution in a test tube. Next, the dilution process is carried out in stages until then the last three series of dilutions are separated to be poured into the media. 0.1 mL of each dilution series was poured into MRS media supplemented with $CaCO_3$, then incubated using an incubator at 37℃ for 48 hours [11].

Bacterial Purification

Colonies that appear with the formation of a clear zone around them were then purified using the quadrant method by streaking them in MRSA media enriched with 1% $CaCO_3$ and then incubating them in an incubator at 37℃ for 24 hours to obtain a single colony. Bacterial purification aims to culture pure bacteria without any contaminants from other bacteria. The next stage was then subcultured with MRSA to become a pure single culture [12].

Identification of Lactic Acid Bacteria

Single colonies that grow after purification were then identified phenotypically (morphologically and biochemically) based on morphological characters observed using macroscopic and microscopic methods. Macroscopic observations consisted of colony shape, margin, elevation and color, while microscopic observations focused on cell shape, color and gram staining [13], as well as biochemical characters which included the bacterial testing process.

Bacterial Gram Staining

Gram staining is carried out by sterilizing the microscope slide using 70% concentrated ethanol, then the bacterial isolate is transferred using a sterile tube and smeared on the surface of the microscope slide which has been treated with distilled water, then homogenized and fixed with the heat of a bunsen burner. After the heat-fixed smear dried, staining was carried out using crystal violet for 1 minute, then rinsed gently with water and air dried. After that, iodine solution (lugol) was added to the dry preparation, left for 1 minute and rinsed with water and then dried in air. After that, the decolorization process was carried out by slowly dripping 96% alcohol onto the preparation until the violet crystals disappeared, then cleaned with water and then dried in air. The preparation was then stained with safranin, left for 1 minute, then washed with water and dried. Next, the bacterial stained slides was observed using a light microscope with 100x magnification [14].

Endospore Staining

The endospore staining process is carried out by homogenizing the bacterial isolate in a microscope slide that has been given distilled water and fixed over the heat of a bunsen burner. Then the malachite green solution was added to the heat-fixed smear, left for 5 minutes, rinsed with water and then dried in air. Next, stained smear was observed using a light microscope with 100x magnification [14].

Biochemical Testing of Lactic Acid Bacteria

Motility Test

The motility test was carried out by inoculating the bacterial isolate vertically on semi-solid MRSA media, then carrying out the incubation process in an incubator at a temperature of 37℃ for 2 days. A positive reaction is indicated by a spreading pattern at the circular needle inoculation point area, while a negative reaction does not show a spreading pattern [15].

Catalase Test

The catalase activity test was carried out by adding 2 drops of 3% H_2O_2 to a 24 hour old bacterial culture on top of a preparation that had been sterilized using 70% alcohol. The formation of oxygen bubbles indicates the activity of the catalase enzyme which can catalyze the reaction of splitting H_2O_2 into H_2O and O_2 [16].

Gas Production Test

The gas production test was carried out by pouring 5 ml of MRSB media into a test tube containing a Durham tube. After that, inoculate the bacterial isolate and place it in an incubator at 37℃ within 48 hours. The presence of trapped air in the form of bubbles and the color of the media changing to yellow in the Durham tube indicates a positive test reaction or formation [17].

Exopolysaccharide Production Test

The exopolysaccharide production test medium was made by weighing 5.22 grams of MRSA medium and 10% sucrose (w/v). Then the media was dissolved with 100 mL of distilled water in a 250 mL erlenmayer, then homogenized and heated until it boiled and the sugar dissolved. After that, the erlenmayer was fitted with a cotton cover, wrapped in plastic and sterilized using an autoclave at 121℃ for 20 minutes [18]. Next, screening for exopolysaccharide-producing lactic acid bacteria was carried out by aseptically growing the bacterial culture in one ose needle on the surface of the media and then incubating for 24 hours at a temperature of 30℃ [19]. Lactic acid bacteria are exopolysaccharide producers if mucus production occurs in the media [20], colonies that are ropy (with strands up to 5 mm long if pierced and pulled with a circular needle) or mucoid (with mucus production even without strands) indicate that the isolate has the potential to produce exopolysaccharides (19). Bacterial isolates with soft textures also show quite high EPS yields, so that apart from being mucoid and roppy, the texture is also soft when observed in EPS-producing bacteria [21].

Data analysis

The research data obtained is presented in the form of a qualitative descriptive method in tabular form, including phenotypic characters (morphological and biochemical identification) and exopolysaccharide characters produced from each isolate of lactic acid bacteria resulting from the marinated fermentation of Pakoba fruit (*Syzygium luzonense* Merr.).

III. RESULTS AND DISCUSSION

RESULTS

Isolation of Acid-Producing Bacteria from Marinated Pakoba (*Syzygium luzonense* Merr.)

Isolation of acid-producing bacteria was carried out on the first and third days of Pakoba marination fermentation, and succeeded in obtaining 12 colonies of acid-producing bacteria which were identified by the presence of a clear zone in the colony growth area on MRSA-$CaCO_3$ media (Table 1). Next, the twelve colonies of acid-producing bacteria were selected/screened to obtain isolates of lactic acid bacteria by carrying out confirmation tests according to the main characteristics of lactic acid bacteria, namely (1) gram staining; (2) cell shape; (3) catalase; (4) spore formation; (5) motility; (6) production of gas or acid from glucose

TABLE 1
COLONY MORPHOLOGY OF ACID-PRODUCING BACTERIA FROM MARINATED OF PAKOBA FRUIT

No	Isolate	Color	Shapes	Elevation	Margin	Inner Structure
1	MP(1) 5.1	Milk white	Circular	Convex	Entire	Opaque
2	MP(1) 5.2	Yellowish white	Circular	Convex	Entire	Opaque
3	MP(1) 6.1	Yellowish white	Circular	Convex	Entire	Opaque
4	MP(1) 6.2	Yellowish white	Circular	Convex	Entire	Opaque
5	MP(1) 7.1	Yellowish white	Circular	Convex	Entire	Opaque
6	MP(1) 7.2	Yellowish white	Circular	Convex	Entire	Opaque
7	MP(3) 6.1	Milk white	Circular	Convex	Entire	Opaque
8	MP(3) 6.2	Yellowish white	Circular	Convex	Entire	Opaque
9	MP(3) 6.3	Yellowish white	Circular	Convex	Entire	Opaque
10	MP(3) 7.1	Yellowish white	Circular	Convex	Entire	Opaque
11	MP(3) 7.2	Yellowish white	Circular	Convex	Entire	Opaque
12	MP(3) 7.2	Yellowish white	Circular	Convex	Entire	Opaque

Based on the colony morphology results, the 12 isolates obtained had a yellowish or milky white, circular, convex, entire and opaque structure in the colony (Table 1).

Selection of Lactic Acid Bacteria

The results of bacterial isolation in Pakoba marination showed that not all colonies that produced acid with clear zones on MRS-$CaCO_3$ media were members of lactic acid bacteria because among the colonies that produced clear zones there were also those that gram-negative, catalase-positive and spore-forming bacteria (Table 2). The selection or screening carried out resulted in 10 of the 12 isolates being identified as lactic acid bacteria with the characteristics of (i) gram positive, (ii) bacilli, (iii) non-catalase (iv) non-spore, (v) non-motile; and (vi) does not produce gas from glucose production.

Exopolysaccharide Test of Lactic Acid Bacteria Isolates

Testing of the exopolysaccharide synthesis of lactic acid bacteria isolates was carried out by observing the character of the extracellular polysaccharides produced by the bacteria. Lactic acid bacteria isolates that have the potential to produce exopolysaccharides are isolates MP(1) 5.2, MP(1) 7.1, MP(3) 6.3 and MP(3) 7.2.

TABLE 2.
SCREENING OF LAB AND NON-LAB ISOLATES GROWING ON MRSA-$CaCO_3$ MEDIA OBTAINED FROM PAKOBA MARINATION

No.	Isolate	Produce Acid (clear zones)	Gram	Catalase	Spore	Motility	LAB / Non-LAB
1	MP(1) 5.1	+	+	+	-	-	Non-LAB
2	MP(1) 5.2	+	+	-	-	-	LAB
3	MP(1) 6.1	+	+	-	-	-	LAB
4	MP(1) 6.2	+	+	-	-	-	LAB
5	MP(1) 7.1	+	+	-	-	-	LAB
6	MP(1) 7.2	+	+	-	-	-	LAB
7	MP(3) 6.1.	+	-	+	-	+	Non-LAB
8	MP(3) 6.2	+	+	-	-	-	LAB
9	MP(3) 6.3	+	+	-	-	-	LAB
10	MP(3) 7.1	+	+	-	-	-	LAB
11	MP(3) 7.2	+	+	-	-	-	LAB
12	MP(3) 7.3	+	+	-	-	-	LAB

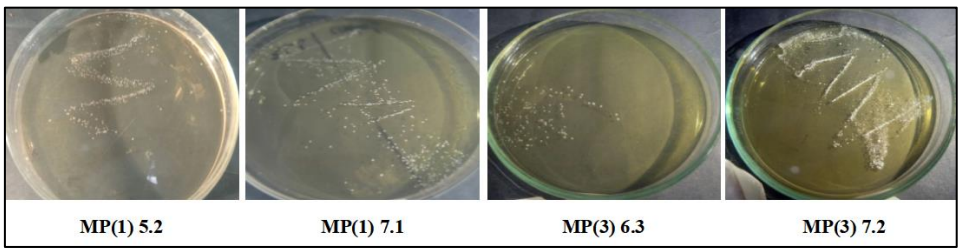


Fig 1. Bacterial Colony Results of the Exopolysaccharide Test of Lactic Acid Bacteria

TABLE 3.
EXOPOLYSACCHARIDE TEST OF LACTIC ACID BACTERIA

Isolate	Characteristics of Bacterial Exopolysaccharides		
	Ropy	***Mucoid***	***Soft***
MP(1) 5.2	-	-	+
MP(1) 7.1	-	-	+
MP(3) 6.3	-	-	+
MP(3) 7.2	-	-	+

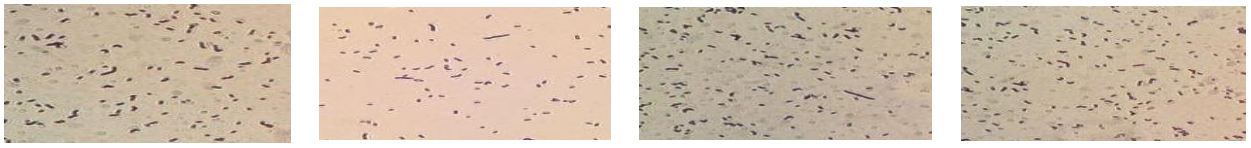
Identification of Exopolysaccharide-Producing Lactic Acid Bacteria from Pakoba marinated

Identification of lactic acid bacteria was carried out on isolates that could produce soft bacterial exopolysaccharides, namely isolates MP(1) 5.2, MP(1) 7.1, MP(3) 6.3, and MP(3) 7.2 using the profile matching method. Based on the results of characterization and genus identification (*generic assignment*) using profile matching (Table 4), 4 isolates of lactic acid bacteria were obtained from Pakoba manrinated samples which were included in the genus *Lactobacillus.* Key characters that differentiate lactic acid bacteria isolates into genus classification include cell shape, cell arrangement, catalase, motility, spore formation and gas production from glucose.

TABLE 4.
GENUS LEVEL IDENTIFICATION (GENERIC ASSIGNMENT) OF LAB ISOLATES ISOLATED IN MARINATED PAKOBA FRUIT BASED ON THE PROFILE MATCHING METHOD

Key Characters*	***Leuconostoc***	***Pediococcus***	***Lactobacillus***	MP(1) 5.2	MP(1) 7.1	MP(3) 6.3	MP(3) 7.2
Cell shapes is bacilli	+	+	-	-	-	-	-
Gram staining	+	+	+	+	+	+	+
Paired cell coniguration	-	+	-	-	-	-	-
Spore formation	-	-	-	-	-	-	-
Catalase reaction	-	-	-	-	-	-	-
Gas production	+/-	-	-	-	-	-	-
Motility test	-	-	-	-	-	-	-
Homofermentatif	-	+	+/-	+	+	+	+
Heterofermentatif	+	-	+/-	-	-	-	-

*Description of key characters of the genera *Lactobacillus, Leuconostoc* dan *Pediococcus* based on *Bergey's manual Systematics of Bacteriology*



(a) (b) (c) (d)

Fig 2. Gram-stained morphology of exopolysaccharide (EPS)-producing lactic acid bacteria (LAB) isolated from marinated Pakoba fruit (*Syzygium luzonense* Merr.). (a) MP(1) 5.2, gram-positive; (b) MP(1) 7.1, gram-positive; (c) MP(3) 6.3, gram-positive; (d) MP(3) 7.2, gram-positive

Discussion

Initial detection of lactic acid bacteria colonies refers to the formation of a clear zone in the colony growth area on MRSA isolation media supplemented with 1% $CaCO_3$ and growing below the surface of the agar medium. Next, the acid-producing bacteria obtained were purified twice using the quadrant streak technique. Next, a screening process was carried out for lactic acid bacteria by confirming the identification of isolates suspected to be lactic acid bacteria through gram reaction, catalase activity, presence of endospores, motility and gas tests.

The results of the isolation of acid-producing bacteria in the fermented Pakoba marinade showed that not all colonies produced clear zones, showing that all the bacteria isolated were members of lactic acid bacteria, because among the colonies that produced clear zones there were also those that showed gram-negative, catalase-positive and spore-forming bacteria (Table 2). These bacteria are suspected to be *E. coli*, Bacillus, Micrococcus, and Pseudomonas. Bacteria in the genus *Bacillus paramycoides, Enterobacter cloacae,* and *Vibrio harveyi* are also bacteria that can produce clear zones or have

amylolytic activity from the amylase enzyme but are not classified as lactic acid bacteria [22].

The 12 isolates were then selected based on the clear zone formed around the colony, and based on confirmation tests as lactic acid bacteria through gram testing, catalase reaction, spore formation, motility, and gas production. 10 isolates were obtained which were lactic acid bacteria. On the first day of fermentation of Pakoba fruit, 5 isolates of lactic acid bacteria were obtained, and on the third day of fermentation of Pakoba fruit, 5 isolates of lactic acid bacteria were also obtained which had a bacilli morphology with a single cell (Table 4). The morphological shape of the cells resulting from the isolation of lactic acid bacteria shows that bacilli bacterial cells are dominant in the fermentation process marinated of Pakoba fruit. Following this, the filtered bacteria were tested for bacterial exopolysaccharides to see whether the lactic acid bacteria isolate obtained from marinated of Pakoba had the ability to produce exopolysaccharides.

Exopolysaccharide testing of lactic acid bacteria isolates was carried out by observing the character of the exopolysaccharide produced by the bacteria. And the results of observations of exopolysaccharide testing showed that four isolates of lactic acid bacteria were capable of producing exopolysaccharides. Two isolates of lactic acid bacteria were obtained on the first day of fermentation and two isolates of lactic acid bacteria on the third day of fermentation which had the ability to produce exopolysaccharides with the property of not producing mucus (moccus) which grew on MRSA media fortified with 10% sucrose. Sucrose acts as an important carbon donor for bacteria in the production of exopolysaccharides. Bacteria use sucrose as fuel for the synthesis of exopolysaccharides which are a type of polysaccharide secreted from cells.

Colony morphology analysis of exopolysaccharide-producing bacterial isolates was carried out through the colony texture identification process using distinctive characteristics as indicators, namely ropy (strands of sticky threads), mucoid (like slimy but not sticky), and soft (soft textured) with convex round colonies and not slimy [21].

Furthermore, lactic acid bacteria that have the potential to produce bacterial exopolysaccharides, (isolates MP(1) 5.2, MP(1) 7.1, MP(3) 6.3, and MP(3) 7.2) were identified using the profile matching method. Based on characterization and genus identification (generic assignment) through profile matching (Table 4), it was found that 4 strains of lactic acid bacteria isolate that had the potential to produce exopolysaccharides obtained during the Pakoba marination fermentation process were classified in the Lactobacillus genus group. The distinguishing characters used to identify the genus of lactic acid bacteria isolates are circular colony morphology and yellowish-white colony color, bacilli morphology, individual cells, gram-positive reaction, non-catalase, and non-spore. This is in line with research that also succeeded in isolating lactic acid bacteria of the genus Lactobacillus from Pakoba fruit through enrichment with a liquid media test method using centrifugation [23].

This genus category is characterized by bacilli, showing gram-positive results because these bacteria are resistant to washing with 96% alcohol and maintain a crystal violet purple color [24], do not produce spores or green when observed with a microscope [25], are not motile or do not propagate in the ose puncture area [23], show non-catalase properties, this can be seen from the absence of gas bubbles when the solution is added [26], The probiotic properties of exopolysaccharides produced from Lactobacillus bacteria can contribute to digestive health by supporting the growth of good microflora in digestion, where bacterial exopolysaccharides can stimulate the immune system, increase good bacteria in the intestine, and act as antioxidants [27]. Characterization of cellular polysaccharides isolated from lactic acid bacteria from marinated of Pakoba has a soft character and can be dissolved in water. This shows that the exopolysaccharide resulting from the fermentation of lactic acid bacteria from marinated of Pakoba has the potential to be used as a food additive that can provide texture and beneficial effects to food products.

CONCLUSION

Lactic acid bacteria (LAB) strains that have the potential to produce exopolysaccharides from isolates of Marination Pakoba (*Syzygium luzonense* Merr.) are isolates MP(1) 5.2, MP(1) 7.1, MP(3) 6.3, and MP(3) 7.2. Strains of lactic acid bacteria that have the potential to produce exopolysaccharides are classified into the genus Lactobacillus based on phenotypic identification through morphological and biochemical tests and can produce soft exopolysaccharides which have the following characteristics: circular, convex, white in color, soft/soft texture and not slimy like the ropy or mucoid type.

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