

Characterization of lactic acid bacteria from local cow's milk kefir

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Abstract. One of products from milk fermentation is kefir. It is made by adding kefir grains which are composed of lactic acid bacteria and yeast into milk. The lactic acid bacteria are a group of bacteria that produce antimicrobial substances and able to inhibit the growth of pathogenic bacteria. In this research, the lactic acid bacteria were isolated from Aceh local cow's milk kefir to determine the genus of the isolates. The methods used in the characterization of lactic acid bacteria are colony morphology, cell morphology, and biochemical tests which includes a catalase test; 5%, 6.5%, and 10% salt endurance tests; 37°C and 14°C temperature endurance tests, SIM test, TSIA test, MR-VP test, and O/F test. Of the four isolates found from the cow's milk kefir, two isolates were confirmed as lactic acid bacteria (isolates SK-1 and SK-4). Both isolates are Gram positive bacteria, and have negative catalase activity. From the observations of colony morphology, cell morphology, and biochemical tests, it was found that the genus of SK-1 is *Lactobacillus* and the genus of SK-4 is *Enterococcus*.

Keywords : characterization, Cow's milk kefir, lactic acid bacteria, morphology,

1. Introduction

Fresh cow milk is one of many beverages with high nutrition, so it is not only benefits to humans but also benefit to decomposers. Bacteria contamination could spread rapidly and cause the quality of the milk goes below average and become no more consumable. To improve the functions, quality and economic factor of milk, managing technique is needed. One of the techniques which are very potential is milk fermentation. Some of the managed milk products which have been developed are fresh milk, yogurt and kefir pasteurization [1].

Kefir is fermented milk product. Kefir can be made by cows', goats' or sheep' milk by adding kefir into one of those milk [2]. White particles are colonies of lactic acid bacteria, such as *Streptococcus* sp., *Lactobacilli* and some types of non pathogen yeast [3]; *Lactobacillus lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and yeast [4]. Lactic acid bacteria are Gram positive bacteria, rod or coccus shaped cell, negative catalase, non-motile and produce lactic acid as the final result of carbohydrate fermentation [5].

Lactic acid bacteria in kefir are proved to have an antibacterial effect against pathogen bacteria that cause food poisoning such as *Salmonella*, *Helicobacter*, *Shigella*, *Staphylococcus* and *Escherichia coli* [6,7]. Food poisoning is a disease caused by consuming contaminated food. Food tends to be contaminated by pathogen bacteria, virus, parasite, natural chemical toxic like poisonous mushrooms



[8]. Food poisoning is a public health problem that happens a lot. Every year 2 million people, especially babies, and toddlers passed away because of food poisoning [9]. *Streptococcus sp.* and *Lactobacilli* which have been isolated from goat's milk kefir can prevent the growth of *Bacillus cereus* [10]. These bacteria cause food to become poisonous because of enterotoxin they produce. Kefir can prevent the growth of *E.coli* with 16.7 mm maximum area of prevention [11].

According to literature study, isolation of lactic acid bacteria from cow's milk kefir, especially in Aceh, have not done yet. Because of that, there is a need to isolate and characterize the lactic acid bacteria from Aceh local cow's milk kefir.

2. Methods

2.1. Instruments

The instruments were used in this study are as follows: petri dish, ose needle (inoculation needle), pipette, micropipette, Erlenmeyer flasks, measuring cylinder, beaker glass, analytical balance, stirring rod, spreader rod, autoclave, magnetic stirrer, test tubes, test tube rack, centrifugation, Bunsen lamps, glass object, glass cover, microscope, oven, refrigerator, incubator, laminar air flow cabinet, hotplate, digital camera, caliper and pencil.

2.2. Materials

The materials were used in this study are as follows : milk of Aceh's cow, kefir seeds from Kazakhstan, distilled water, alcohol 70% and 95%, KOH 3%, alpha-naphtol, Kovacs reagent, methyl red reagent, crystal violet, iodine, safranin, hydrogen peroxide (H₂O₂) 3%, physiological NaCl, 5% NaCl, 6.5% and 10%, de Mann Rogosa Sharpe agar (MRS agar) media, triple sugar iron agar (TSIA) media, sulfide indole motility (SIM) media, methyl red and Vogue Proskauer (MR-VP) media, oxidative/fermentative (O/F) media, and nutrient agar (NA) media.

2.3. Instruments and Materials Sterilization

All instruments washed until clean and dried. Then the instruments and materials were sterilized using an autoclave at a temperature of 121°C and a pressure of 15 psi for 20 minutes.

2.4. Milk Fermentation

The raw material is fresh milk kefir. Milk used in this study was the milk of Aceh's cow from the dairy farm. A total of 1 L of pasteurized cow's milk must be cooled at room temperature (28°C) and added by kefir seeds of Kazakhstan. Cow's milk was incubated at room temperature for 20-24 hours. Then, the milk became thick and formed cow's milk kefir.

2.5. Lactic Acid Bacteria Isolation

Isolation of lactic acid bacteria was done by suspending 1 ml kefir of fermented cow's milk into 9 ml of distilled water (dilution 10⁻¹). Then, 1 ml sample from dilution 10⁻¹ was taken to dissolved in 9 ml of distilled water (dilution 10⁻²), and so on, until the dilution 10⁻⁷. Then 1 ml sample was taken from each dilution and grown in de Mann Rogosa Sharpe agar (MRS agar) media with the spread plate method. Furthermore, the media were incubated at 37°C for 24 hours. Isolates which were grown then retrieved and inoculated on MRS agar plate using ose needle and streak method to obtain pure cultures was incubated for 24-48 hours at 37°C in the incubator.

2.6. Lactic Acid Bacteria Characterisation

2.6.1. Morphological Observation of Lactic Acid Bacteria Colonies

Observations of colony morphology was done after getting a pure culture in a petri dish. These observations were made visually include shape, color, edges, and the elevation of bacterial colonies. The surface of colonies can be seen from the side and the edge of the colony can be seen from the above [12].

2.6.2. Morphological Observation of Lactic Acid Bacteria Cells (Gram Staining)

Object glass slides that will be used for Gram staining technique was sterilized by 70% alcohol. Furthermore, one of bacteria isolates was taken by use needle aseptically and smeared on a slide and fixed several times on the Bunsen lamp fire. Then bacteria isolates were etched with two drops of crystal violet and allowed for 1 minute. Furthermore, isolates were washed with distilled water and dry-aired. Then, iodine was dropped on it and allowed for 1 minute, and isolates were washed with distilled water and dry-aired. Bacteria isolates were mixed with drops of 95% alcohol slowly for 30 seconds, then isolates were washed with distilled water and dry-aired. Isolates then spilled by safranin slowly for 30 seconds. Then, isolates were washed with distilled water and then dry-aired again. Bacteria isolates preparates were observed using a microscope. Observations included the color and shape of a bacterial cell. Gram-positive bacteria marked by the purple (or blue) color which indicates that the bacteria cell wall are capable of binding to crystal violet dye, whereas Gram-negative bacteria is characterized by the formation of pink color indicates that the bacteria cell wall are not able to bind the crystal violet dye, and only stained by safranin dye (as comparative dye).

2.6.3. Biochemical Tests of Lactic Acid Bacteria

2.6.3.1. Catalase Test

Catalase test was conducted by dripping two drops of hydrogen peroxide (H₂O₂) 3% at 24 hours aged cultures on an object glass slide. Catalase test positive reaction characterized by the formation of oxygen bubbles that indicate the bacteria produce the catalase enzyme which converts H₂O₂ to water (H₂O) and oxygen (O₂) [12].

2.6.3.2. Growth Test on Different Concentrations of Salt

One colony of bacteria was inoculated into the de Mann Rogosa Sharpe broth (MRS broth) media with a concentration of 5%, 6.5% and 10% NaCl. Then the cultures were incubated at 37°C for seven days. The bacteria growth showed by the sediment formation in the media.

2.6.3.3. Growth Test at Different Temperatures

One colony of bacteria was inoculated into the de Mann Rogosa Broth (MRS broth) media and incubated at temperatures 14°C and 37°C for seven days. The bacteria growth showed by the sediment formation in the media.

2.6.3.4. Motility Test

One colony of bacteria inserted into the sulfide indole and motility (SIM) media in a test tube. The test tube incubated for 48 hours at 37°C. The observation of motility test is the growth of bacteria on the media. The bacteria that only grow around inserted location show a negative result, while the bacteria that grow on the media surface or spread in the media show positive result [13].

3. Results and Discussions

Four isolates of milk kefir (SK-1, SK-2, SK-3, SK-4) were obtained from the dilution method. Dilutions of 10⁻¹ until 10⁻⁷ of kefir were grown on selective media for lactic acid bacteria, de Mann Rogosa Sharpe agar (MRS agar).

3.1. Morphological Characterization of Lactic Acid Bacteria

Characterization is the basis for bacterial identification. Characterization can be done based on colony morphology, characteristic of cytology (cell type and Gram staining) and physiological characteristics [16]. Observed morphology in this study was colony morphology and cell morphology. Colony morphology includes color types, edges, and elevation of colonies that observed visually, while the cell morphology was observed by Gram stain using a microscope.

3.1.1. Colony Morphology

Colony morphology of 4 isolates (SK-1, SK-2, SK-3 and SK-4) bacteria are round (circle) shape (100%), has slippery (smooth) edge (100%), the form of convex elevation (100%), and milky white (75%) and cream (25%) of colony color (Table 1). Several isolates of lactic acid bacteria isolated from fresh goat's milk have circle type colonies, smooth edges, convex elevation and a milky white color [17].

Table 1. Characteristics of colony morphology and cell morphology of isolates

No.	Isolate Code	Colony Morphology				Cell Morphology	
		Type	Color	Edge	Elevation	Cell Shape	Gram Staining
1	SK-1	Circle	Milky White	Smooth	Convex	Bacilli (rod)	Positive
2	SK-2	Circle	Milky White	Smooth	Convex	Bacilli (rod)	Negative
3	SK-3	Circle	Milky White	Smooth	Convex	Bacilli (rod)	Negative
4	SK-4	Circle	Beige/Cream	Smooth	Convex	Cocci (spherical)	Positive

3.1.2. Cell Morphology

Cell morphology was done to determine the colored smear culture under a microscope and see how the shape of the cell, the characteristic of the Gram bacteria [18]. Gram staining results showed that isolates SK-1 and SK-4 are purple. These results showed that isolate SK-1 with rod shape of cell and SK-4 with cocci shape of cell belonging to the group of Gram-positive bacteria. While the results of isolates SK-2 and SK-3 are red. It shows these isolates belong to the group of Gram-negative bacteria with the rod shape of cell (Table 1). Isolates SK-1 and SK-4 with Gram-positive results included in the lactic acid bacteria group, whereas isolates SK-2 and SK-3 with Gram-negative results are not included in the group of lactic acid bacteria. These results are supported by researches [19, 20] which states that the lactic acid bacteria (LAB) are in the group of Gram-positive with the shape of cell cocci or bacilli. Research [21] also found five Gram-positive isolates that belong to the lactic acid bacteria, and five isolates of Gram negative excluding lactic acid bacteria.

3.2. Biochemical Tests

The biochemical test is a method to identify pure cultures of bacteria isolates through the characteristic of cell physiology. Biochemical tests performed in this study include catalase test, salt resistance test, test temperature resistance, SIM test, TSIA test, MR-VP test, and O/F test. The results of biochemical tests of bacteria isolated from milk kefir can be seen in Table 2.

Table 2. The biochemical tests' results of cow's milk kefir LAB isolates

No.	Biochemical Test	Isolate Code	
		SK-1	SK-4
1.	Catalase	-	-
Salt endurance			
2.	- 5%	+	+
	- 6,5%	+	+
	- 10%	+	+
Temperature endurance			
3.	- 14°C	+	+
	- 37°C	+	+
4.	Motility	-	-
5.	Indol	-	-
6.	B/S	Y/Y	Y/Y
	Gas	-	-
	H ₂ S	-	-
7.	Sucrose	+	+
8.	Lactose	+	+
9.	Glucose	+	+
10.	MR	+	+
11.	VP	-	-
12.	O/F Test	F	F
Identification result		<i>Lactobacillus</i>	<i>Enterococcus</i>

Information :

+ : Positive reaction - : Negative reaction
 B : Butt S : Slant
 Y : Yellow F : Characteristically fermentative

3.2.1. Catalase Test

Lactic acid bacteria do not produce catalase enzyme that converts hydrogen peroxide into water and oxygen. Some bacteria require oxygen to form hydrogen peroxide, which is a byproduct of aerobic metabolism that is toxic. While lactic acid bacteria do not require oxygen, because it includes in groups of anaerobic bacteria (bacteria that do not need oxygen to grow) [22, 23]. Catalase reaction is positive when the formation of air bubbles that indicate the formation of O₂ gas, and negative catalase results if it does not indicate the presence of gas bubbles. These bacteria are not able to produce the catalase enzyme to convert hydrogen peroxide / H₂O₂ into water and oxygen, which is one of the characteristics of lactic acid bacteria [24]. Catalase is an enzyme that serves to decompose hydrogen peroxide into water and oxygen (2H₂O₂ + O₂) [25]. Research [26] found nine isolates were negative catalase lactic acid bacteria isolated from fermented goat milk.

3.2.2. Salt Endurance Test

Salt endurance test was done to determine the ability of bacterial growth at different salt concentrations. Salt endurance test results showed that isolates SK-1 and SK-4 may grow at the concentrations of 5%, 6.5%, 10% NaCl. Lactic acid bacteria that were grown included in the BAL group of moderately halophilic and extremely halophilic namely the need of salt for growth at a concentration of 5-30% [27]. Each genus of lactic acid bacteria has different tolerances to grow on media with different concentrations of NaCl salt [28]. Lactic acid bacteria of the genus *Lactobacillus*, *Pediococcus* and *Leuconostoc* are able to grow and thrive on fermented using high salinity [29].

Research [30] found four isolates of lactic acid bacteria that can live at NaCl concentration of 4% and one isolates at a concentration of 6.5% NaCl isolated from fermented milk.

3.2.3. Temperature Endurance Test

Temperature endurance test was done to determine the ability of bacterial growth at different temperatures. The test results showed the isolates SK-1 and SK-4 were able to survive at different temperatures. Both isolates can grow at temperatures of 14°C and 37°C. The bacterial growth showed by sediment formation in the media. Lactic acid bacteria are classified as mesophilic bacteria growth with a temperature range between 10-45°C [31]. According to [32, 33] the optimum temperature for growth of lactic acid bacteria is 30-45°C. This result is supported by research [34], getting three isolates of lactic acid bacteria from fermented goat's milk that can grow at a temperature of 14°C, but the better growth seen at 37°C. Each genus of lactic acid bacteria has different optimum growth temperature [28].

3.2.4. Motility Test

The aim of motility test is to determine the bacteria are motile or non motile, if there are flagella which function to use in movement. Motility testing of isolates SK-1 and SK-4 showed results are non motile. Isolates SK-1 and SK-4 are categorized as non motile because the growth was only along the line of inoculation. This result suggests that all the LAB isolates do not have flagella. Flagella is a filamentous structure composed of proteins attached to the cell surface and allows the movement of motile bacteria. Motility test is positive if the growth of the colony spreaded on agar [35]. Lactic acid bacteria isolates from buffalo milk curd is non motile [36]. Lactic acid bacteria of the genus *Lactobacillus* has a characteristic of non motile bacteria [35].

4. Conclusion

Based on analysis of the results, it can be concluded that the results of isolation and characterization of lactic acid bacteria from Aceh local cow's milk kefir got two isolated bacteria. Isolate SK-1 is bacteria from *Lactobacillus* genus and isolate SK-4 is *Enterococcus* genus. Isolate SK-1 identified as lactic acid bacteria *Lactobacillus* genus with the characteristics are Gram positive, non-motile, rod shaped cell, negative catalase, and other physiological characters based on the biochemical tests. Isolate SK-4 identified as lactic acid bacteria *Enterococcus* genus with the characteristics are Gram positive, non-motile, coccus shaped cell, negative catalase, and other physiological characters based on the biochemical tests.

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