# ISOLATION, CULTURE AND CHARACTERIZATION OF LACTIC ACID BACTERIA FROM KEFIR GRAINS FERMENTED MILK

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## Abstract

Kefir or kephir is a fermented milk drink similar to a thin yogurt that is made from kefir grains, a specific type of mesophilic symbiotic culture. Home-made kefir has been improved using various natural substrates such as milk, coconut water, and fruits such as grape, apple and dragon fruits. The present study includes isolation, culture and characterization of lactic acid bacteria from milk kefir. The isolated bacteria strain was characterized by gram staining and biochemical tests. The observed background color of the selected isolated bacteria strain is violet for gram staining, the isolated bacteria strain may be gram positive. From direct isolation from milk kefir, the isolated bacteria were negative for motility, indole, gelatin, citrate utilization, catalase, Voges-Proskauer, nitrate reduction, urease, starch hydrolysis tests, sugar fermentation test and methyl red test. For the result obtained, the most of biochemical tests for all bacteria slants were agree with reported literature data for lactic acid bacteria.

**Keywords:** kefir, lactic acid bacteria, fermentation, milk, biochemical tests

## Introduction

Microorganisms play an essential role in the food fermentations. Lactobacillus is also formed in some fermented foods like yogurt and in dietary supplements. Fermentation is one of the oldest and most economical method used in food preservation. The properties of fermented milks (curd, yoghurt, kefir, Kumis, etc.) with their nutritional values have driven a considerable interest (Lim *et al.*, 2007).

Kefir is an alcoholic, fermented milk beverage produced by the fermentation of kefir grains, which contain lactic acid bacteria, acetic acid bacteria, and yeasts. It originated from Caucasus mountain in former Soviet Union, central Asia. Milk Kefir is made with cow milk, goat milk, or coconut milk. Starter culture is prepared from kefir grains, *Lactobacillus kefiranofaciens*, and species of the genera *Leuconostoc*, *Lactococcus*, and *Acetobacter* growing in a strong specific relationship. Kefir has a tart, creamy flavor, and it is loaded with probiotic health benefits. It is safe for most people to consume, and a single serving is full of vitamins and probiotics. It is safe to consume daily, and it may help create and maintain a healthy balance of good bacteria in multiple systems within the body (Farnworth, 2005).

Probiotic bacteria found in kefir products include: *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus kefiranofaciens*, *Lactococcus lactis*, and *Leuconostoc* species. Lactobacilli in kefir may exist in concentrations varying from approximately 1 million to 1 *billion* colony-forming units per milliliter, and are the bacteria responsible for the synthesis of the polysaccharide *kefiran* (Oliveira *et al.*, 2013).

Lactic acid bacteria comprise a group of bacteria that are united by a constellation of morphological, metabolic and physiological characteristics. The genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* are important members of this group (Batt, 2000). Lactic acid bacteria are an order of gram-positive, either rod-shaped (bacilli) or spherical (cocci) bacteria that share common metabolic and physiological characteristics. These bacteria, usually found in decomposing plants and milk products, produce lactic acid as the major

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metabolic end product of carbohydrate fermentation. The lactic acid bacteria are found in foods (dairy products, fermented meat, sour dough, fermented vegetables, beverages- including wine and kefir). Health benefits of lactic acid bacteria are known to give positive influence in the gastrointestinal of humans (Chen *et al.*, 2010).

In the present research work, the lactic acid bacteria were isolated from milk kefir by direct isolation method and isolated bacteria strains were identified by gram staining method and biochemical tests (Breed *et al.*, 1957).

## Materials and Methods

Firstly, sampling of milk kefir grains and processing of milk kefir were performed. Then, the lactic acid bacteria were culture by serial dilution and isolated after fermentation. The isolated bacteria were characterized by gram staining method and biochemical test. In this study, microbiological work was conducted in the Fermentation Department, Pharmaceutical Research Department, Ministry of Industry 1, Yangon Region.

### Sample Collection

Milk kefir grains (Figure 1) was purchased from the NIHON KEFIA Co., Ltd, Japan.

**Figure 1** Milk kefir grains

### Processing of Milk Kefir

Milk kefir grains (ca. 4 g) were added in a jar and then fresh milk (250 mL) was added. The glass jar was sealed with clean clothes and allowed to culture for 12 to 48 h at room temperature, then the kefir grains were removed, resulting the milk kefir recipes (Figure 2).

**Figure 2** Preparation of milk kefir recipes

### Tomato juice medium

About 2 g of agar, 1 g of yeast extract, 1 g of dextrose, 0.05 g of dipotassium phosphate, 0.02 g of monopotassium phosphate, 0.02 g of magnesium sulphate, 0.001 g of manganese sulphate, 0.001 g of ferrous sulphate, 0.001 g of sodium chloride, 2 mL of tomato juice were mixed with 100 mL of distilled water (pH 6.7). The mixture was boiled on hot plate, sterilized in sterilizer (121˚C), cooled and transferred 20 mL to each petridish (Atlas, 1993).

### Isolation of *Lactobacillus* Species by Serial Dilution Method and Streaking Method

One gram of sample was added into a conical flask containing 99 mL of sterile distilled water to make a dilution ratio of 1:100. The mixture was shaken for about 5 min. 1 mL of each serially diluted solution was added to 9 mL of sterilized distilled water for each sample to conduct bacteriological analysis. After four serial dilutions, 1 mL of each dilution level was inoculated immediately on to sterile petri dishes containing 25 mL of Rogosa and tomato juice agar media inside a clean bench under laminar flow. Each dilution was prepared in two sets, one for heterotrophic viable count and another for isolation and identification. The inoculated plates were isolated in clockwise and anti-clockwise directions to distribute the inoculums on the surface the medium and incubated at 37 °C for 24-72 h. The colonies that developed on the inoculated plates were observed under microscope and streaked selectively as pure strain to new set of petri dishes containing the same Rogosa and tomato juice agar media and, incubated at 37 °C for 24-72 h, bacteria slant cultures were obtained. From these culture slants, MK-1 and MK-2 were selected. (Collin *et al.*, 1995; Dubey and Maheshwari, 2002).

### Identification of the Isolated *Lactobacillus* Species

Identification of each isolate (MK-1 and MK-2) of bacteria up to genus level was carried by the gram staining method and biochemical tests.

### Determination of Staining Characteristics by Gram's Stain

Preparations for staining were made on microscopic slides, which were cleaned by immersion in chromic acid and then washing with water. The clean slide was held with a pair of forceps and dried by passing through the flame of spirit burner. A drop of sterile distilled water was placed on the perfectly clean slide. A loopful of pure isolated colony from plate was taken with a sterile inoculating loop and mixed with sterile distilled water on the slide. Subsequently the slide was dried by passing quickly through the flame of a spirit burner. The slide was then flooded with crystal violet solution for 1 min, washed thoroughly under tap water, and then smeared with iodine solution for 1 min. It was then decolorized with acetone/alcohol and washed with water. Counter staining with safranin was done for about 20-30 s and washed with water and dried by blotting paper and examined under a compound microscope for cell morphology.

### Biochemical Tests

#### Motility test

Motility stab agar was prepared and inoculated with isolated bacteria for 2 days at room temperature. If the tube was turbid, this indicated that isolated bacteria were motile (Atlas, 1993).

#### Citrate utilization test

A loopful of isolated bacteria was inoculated into the surface of citrate slant medium by even spreading and incubated at 27 °C for 5 days. After this period, the appearance of a blue colour on the citrate slant agar medium indicated a positive citrate utilization test (Cruickshank *et al.*, 1968).

#### Indole test

The isolated bacteria were inoculated in the peptone water medium and incubated at room temperature for 48 h. After this period for the occurrence of indole reaction, 0.5 mL of Kovac's reagent was added to the test tubes and was shaken gently. If the pink layer could occur within a few seconds in the alcoholic layer, the indole test was positive and a yellow layer develop and the indole test was taken as negative one.

**Nitrate reduction test**

A loopful of isolated bacteria was inoculated into the nitrate medium and incubated for 96 h at 27 °C. After incubation, one drop each of the test reagent was added to the test culture. A red colour developing within a few min was indicative of a positive nitrate reduction test (Cowan, 1974).

**Methyl red test**

Glucose phosphate peptone broth medium was inoculated and incubated at room temperature for 48 h. It is detecting on organisms that does not convert acidic products to neutral products and produces final pH lower than that of organisms producing neutral products. Because of lower pH, the addition of methyl red indicator changes to a red colour as positive reaction (Bisen and Verma, 1998).

**Voges-Proskauer test**

After autoclaving, the solution was cooled and 5 mL of sterile glucose solution added quickly to it near the flame of a spirit burner to get glucose phosphate peptone-water medium. The medium was distributed to each of the sterile test tubes in 5 mL amounts. A loopful of isolated bacteria was inoculated into the glucose phosphate peptone-water medium and incubated at 24 °C for 48 h. After incubation for Voges-Proskauer reaction, 1 mL of 40 % potassium hydroxide solution and 3 mL of 5 % α-naphthol in absolute ethanol solution were added to the test culture and shaken quickly. The development of a pink colour in 2 to 5 min was indicative of a positive VP test (Cruickshank *et al.*, 1968).

**Gelatin liquefaction test**

A loopful of bacteria was inoculated into gelatin agar medium and incubated at 27 °C for 72 h. Then, they were stored in refrigerator for 30 min. If the medium was liquid, they would be positive in gelatin liquefaction.

**Catalase test**

A few drops of 3 % hydrogen peroxide ($H_2O_2$) solution was added onto each slide containing strain and watched for immediate signs of bubbling, which represented positive test; absence of bubbles indicated a negative test (Salle, 1948).

**Urease test**

The broth medium is inoculated with a loopful of a pure culture of the test organism and incubated the test tube at 37 °C for 48 h. The phenol red indicator will turn to pink due to alkaline nature of the medium because of ammonia production (Dubey and Maheshwari, 2002).

**Starch hydrolysis test**

Isolated bacteria were streaked on starch agar medium and allowed it to grow at 37 °C for 48 h. Iodine solution was poured on the plates. If the area around streaked culture remains clear it indicated the degradation of starch had occurred due to production of amylase (Dubey and Masheshwari, 2002).

**Sugar fermentation test**

Fermentation medium (10 mL) containing 1 % each of sugars such as glucose, lactose, maltose and sucrose were separately added into the test tubes and each with an inverted Durham

tube. The medium was sterilized at 121 °C for 15 min, which were then inoculated with isolated bacteria and incubated for 48 h. (Cruickshank *et al.*, 1968).

## Results and Discussion

The results and discussion consist of two parts. The first part concerned with isolation of *Lactobacillus* species from milk kefir sample by serial dilution method. The second part includes the identification of *Lactobacillus* species from milk kefir sample by gram staining method and biochemical tests.

### Isolation and Identification of *Lactobacillus* Species from Milk Kefir Sample

### Isolation of *Lactobacillus* species from milk kefir sample

In the dilution method contained in the procedure, single colonies of isolated bacteria appeared on each of the four tomato juice agar plates with different dilution after incubation for 4 days (Figure 4). The colony from each dilution (plate) was transferred to each tomato juice agar slant for culture.

**Figure 3** Photograph showing the isolation and culture of *Lactobacillus* species from mil kefir

(i) Streaking for isolation of *Lactobacillus* species colony

(ii) Slant culture of isolated *Lactobacillus* species

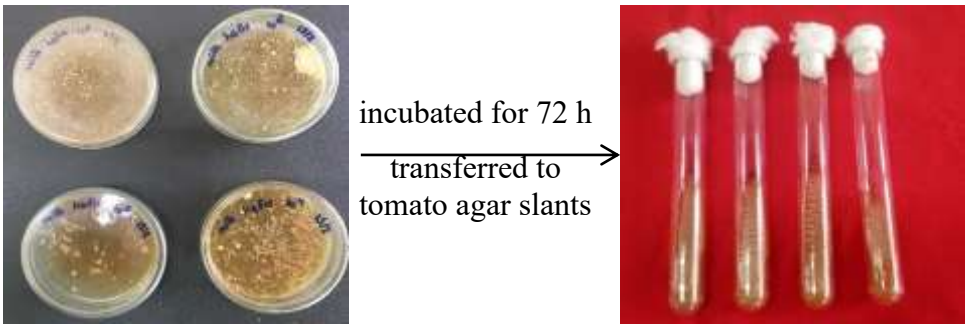
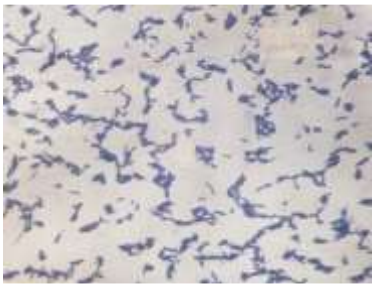


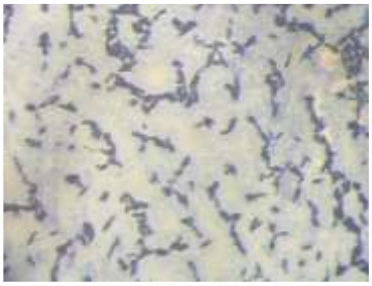

**Figure 4** Photograph showing the single colony of isolated bacteria from milk kefir by dilution method

**Identification of *Lactobacillus* species from milk kefir sample**

**Gram staining**



Isolate MK-1



Isolate MK-2

**Figure 5** Gram staining for bacteria strain isolated from milk kefir sample

The isolation and identification of *Lactobacillus* species from milk kefir were conducted. Two bacterial strains (MK-1 and MK-2) from 24 h incubation period was isolated, purified and identified. The colony character of isolates (MK-1) was cream colour, circular shape and MK-2 was creamish-white color and irregular shape.

The observed background colour is violet for gram staining for bacteria strain isolated from MK-1 and MK-2 sample. Therefore, the isolated bacteria were gram positive bacteria.

**Biochemical characteristics of *Lactobacillus* species from milk kefir sample**

The identification of genus level, two isolated bacteria were carried out by biochemical tests. All isolated *Lactobacillus* species from milk kefir were negative results in motility test, indole test, methyl red test, gelatin liquefaction test, citrate utilization test, nitrate reduction test, catalase test, Voges-Proskauer test, urease test and starch hydrolysis test. These results were in accordance with those revealed in Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1957). From the result of biochemical tests, the most of biochemical tests for all bacteria slants were agree with the reported literature data for lactic acid bacteria.

These results are shown in Table 1.

**Table 1 Biochemical Characteristics of *Lactobacillus* Species from Milk Kefir Samples**

| Bacteria strain | Biochemical Tests | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Motility | Indole | Methyl red | Gelatin Liquefaction | Citrate utilization | Catalase | Voges-Proskauer | Urease | Starch hydrolysis |
| MK - 1 | - | - | - | - | - | - | - | - | - |
| MK - 2 | - | - | - | - | - | - | - | - | - |

+ = positive reaction, - = negative reaction

In the sugar fermentation test (glucose, lactose, maltose and sucrose) were used. All isolate showed negative results. These results were also similar to the result showed in Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1957). The results of sugar fermentation test are presented in Table 2.

**Table 2 Sugar Fermentation Tests of *Lactobacillus* Species from Milk Kefir Samples**

| Bacteria strain | Sugar test | | | |
|---|---|---|---|---|
| | Glucose | Maltose | Lactose | Sucrose |
| MK-1 | - | - | - | - |
| MK-2 | - | - | - | - |

## Conclusion

In the present study, the selected bacteria strains were isolated by using tomato juice agar medium from milk kefir samples. These strains were identified by biochemical characteristics using Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1957). From the study of identification of isolated bacteria, the observed background color of selected isolated bacteria strain is violet for gram staining reaction showed the isolated bacteria is gram positive bacteria.

From the biochemical tests, all isolated *Lactobacillus* species provided negative results in motility test, indole test, methyl red test, gelatin liquefaction test, citrate utilization test, nitrate reduction test, catalase test, Voges-Proskauer test, urease test and starch hydrolysis test. From the result of biochemical tests, the most of biochemical tests for all bacteria slants were agree with reported literature data for lactic acid bacteria.

In the results of sugar fermentation tests in milk kefir samples, all isolates were found to ferment glucose, lactose, maltose and sucrose. According to the gram staining and biochemical tests, the isolated bacteria is lactic acid bacteria.

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