



## The characteristics of lactic acid bacteria isolated from Indonesian commercial kefir grain

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### ABSTRACT

**Aims:** Kefir is a fermented dairy product using lactic acid bacteria, such as *Lactobacillus lactis* and *L. delbrueckii* subsp. *bulgaricus* mixed with yeast and produces acids and alcohols. Lactic acid bacteria (LAB) can be identified based on morphological and biochemical characteristics, using macroscopic and microscopic examination and biochemical tests. This study aims to isolate LAB from commercial kefir grain and to conduct an identification based on morphological characteristics using macroscopic, microscopic and biochemical techniques.

**Methodology and results:** LAB isolated from kefir grain was grown on nutrient-agar medium and then transferred to the MRS agar medium. Macroscopic observations included recording the colony size, color, form, surface, and edge. Microscopic examination involved Gram staining and spore staining. Biochemical assays included oxidase test and aerobic and anaerobic tests. The results obtained were lactic acid bacteria which were negative in the catalase and oxidase tests, positive for Gram staining, spore staining was negative, and aerobic and anaerobic tests were positive; the bacteria were rod-shaped and rounded.

**Conclusion, significance and impact of study:** Lactic acid bacteria isolated from kefir grains were identified by macroscopic and microscopic examination of morphology and using biochemical tests putatively identified as *Lactococcus cremoris*, *Streptococcus cremoris* and *L. plantarum*.

**Keywords:** Characteristics, insulation, kefir grain, lactic acid bacteria

### INTRODUCTION

Fermentation organisms are very varied, for example, lactic acid bacteria (LAB) in dairy products (Bamforth, 2005). LAB is classified into several genera including *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Lactobacillus* (Goldin, 1998). One of the products of fermentation utilizing LAB is kefir. Kefir is a fermented dairy product using the LAB, such as *L. lactis* and *L. delbrueckii* subsp. *bulgaricus*, and yeast, and produces acids and alcohols. Gaware *et al.* (2011) reported that a fundamental difference between yogurt and kefir lies in the type of bacteria used. Yogurt consists of beneficial bacteria, but kefir contains several strains of bacteria that are not found in yogurts, such as *L. caucanus*, *Leuconostoc*, *Aerobacter*, and *Streptococcus*.

The identification of LAB can be conducted by observation of morphological and biochemical properties *in vitro*. Morphological characteristics observed at macroscopic examination include colony size, color, form,

edge, and surface. The microscopic examination involves staining techniques, such as Gram staining, and observation of the shape and size of the cells. Biochemical techniques include catalase, oxidase, aerobic and anaerobic tests (Holt *et al.*, 1994). Lactic acid bacteria are naturally present in milk and other dairy products, such as milk of Sumbawa horse (Sujaya *et al.*, 2008), sheep milk (Acurcio *et al.*, 2014), cow milk (Wassie and Wassie, 2016), buffalo milk (Duary *et al.*, 2011; Rizqiyati *et al.*, 2015), dadiah (Sameen *et al.*, 2010), cheese (Zeppa *et al.*, 2004; Abdi *et al.*, 2006; Luiz *et al.*, 2017), yoghurt (Azadnia and Khan Nazer, 2009; Ali, 2011; Khalil and Anwar, 2016;) and kefir grain (Sefidgar *et al.*, 2014; Zanirati *et al.*, 2014; Garofalo *et al.*, 2015; Kivanç, 2015).

There is limited information available on LAB from Indonesian commercial kefir, although this information is important for improving the quality of kefir production.

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Based on this background, it is considered necessary to conduct research on the isolation and identification of LAB from commercial kefir grain. This study aims to isolate and identify LAB from commercial kefir grain based on morphological and biochemical characteristics using macroscopic, microscopic and biochemical techniques.

## MATERIALS AND METHODS

### Rejuvenation (activation) of kefir grain

Kefir Grain Growth of commercial kefir advance rejuvenated (activated) with a 10% reconstituted milk medium, incubation for 24 h. Kefir grains were then filtered and incubated in 10% reconstituted milk medium for 24 h at 37 °C. The kefir grains were then filtered and grown on Nutrient Agar (NA) medium.

### Isolation and purification of lactic acid bacteria

Activated kefir grains then growth on a Petri dish containing NA media with a quadrant streak method and casting method dilution 10:1, 10:2, 10:3, 10:4 and 10:5 were incubated for 24 h at 37 °C. Bacterial colonies that demonstrated growth were then transferred to Petri dishes containing de Man, ROGOSA, Sharpe Agar (MRS agar) using a quadrant streak method and incubated for 24 h at 37 °C. Purification of isolates was conducted by selecting colonies with a dominant colony morphology with characteristics different to other colonies. Isolated colonies were inoculated onto solid media using the streak method. Purified isolates were then grown on an agar slant and analyzed using macroscopic and microscopic examination and biochemical assays.

### Identification of lactic acid bacteria contains in kefir grain

Inoculated bacteria colonies are streak quadrant, poured on MRS agar, and incubated at 37 °C for 24 h. Macroscopic observations recorded included the size of the colony, color, form, margin and surface edge. Microscopic examination firstly involved Gram staining (Madigan *et al.*, 2010) to show the cell shape and size. Staining of spores (Lay, 1994) was then conducted. Observations were recorded using a Zeiss Microscope Imager Asio A2 and camera (Zeiss AxioCam Hrc, Germany).

### Biochemistry test

Biochemical tests were performed on bacterial colonies, such as oxidase (Harrigan, 1998), catalase (Harrigan, 1998), aerobic and anaerobic, and motility tests (Rahayu and Margino, 1997).

### Temperature resistance test

One LAB colony was added to MRS Broth (MRSB) and then incubated at temperatures of 10, 37 or 50 °C for 2-5 d. Positive results were indicated by the presence of turbidity in the MRSB media (Harrigan, 1998).

## RESULTS AND DISCUSSION

### Isolation and identification of lactic acid bacteria

Three isolates of the LAB were isolated from plain kefir grain, namely I<sub>1</sub>, I<sub>2</sub>, and I<sub>3</sub>. Isolation of lactic acid bacteria aimed to determine and ensure the genus of bacteria found in kefir grains. Macroscopic and microscopic observations of the three isolates of the LAB that have been isolated and grown on MRS agar are shown in Figure 1 and characterization of the isolates in Table 1.

**Table 1:** Macroscopic observations of lactic acid bacteria isolated from Indonesian commercial kefir grains.

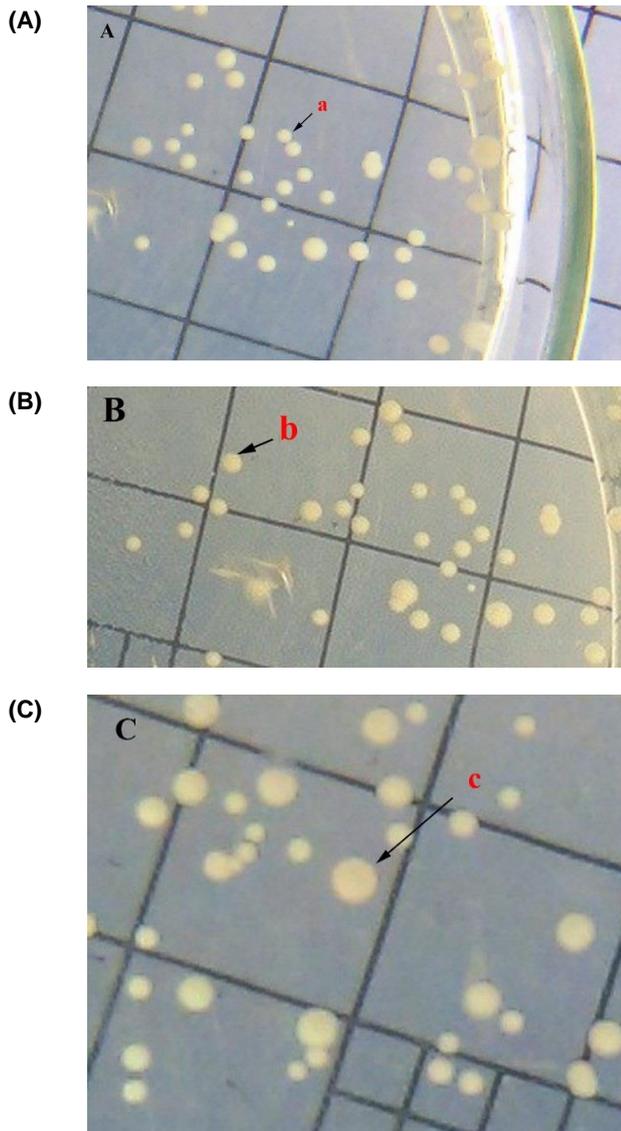
Observations	Bacterial Isolates		
	I <sub>1</sub>	I <sub>2</sub>	I <sub>3</sub>
Size of the colony (mm)	0.5-1 mm	0.5-1 mm	0.5-1 mm
Size of the colony (description)	Small	Medium	Large
Form of the colony	Circular	Circular	Circular
Margin	Entire	Entire	Entire
Elevation	Convex	Convex	Convex
Colony color	White	White	White
Behavior beyond light	-	-	-

Figure 1 shows visible growth of LAB on MRS agar. MRS agar is a selective medium used for the growth of LAB. These results suggest that kefir grain starter in isolation including lactic acid bacteria. Zahoor *et al.* (2003) suggest that MRS is the most common medium used for growing LAB, including the genera *Lactobacillus*, *Streptococcus*, *Pediococcus*, and *Leuconostoc*.

Based on macroscopic observations, there are three types of colonies grown on MRS agar: (A) I<sub>1</sub> isolates formed small colonies, (B) I<sub>2</sub> isolates formed medium size colony and (C) I<sub>3</sub> isolates formed large size colonies. Three races were then grown on MRS agar slants to be used as a mother starter. The detailed results of macroscopic observations of bacteria isolates I<sub>1</sub>, I<sub>2</sub> and I<sub>3</sub> are shown in Table 1.

Table 1 includes macroscopic observations of bacterial isolates, showing that I<sub>1</sub> has a diameter size of 0.5-1 mm, has a circular shape with the edge of the colony of entire colonies, a convex surface, and a milky-white color. The same observation was seen in isolates I<sub>2</sub> and I<sub>3</sub>, although in isolates I<sub>3</sub> colony size appears larger than I<sub>1</sub> and I<sub>2</sub>. Sameen *et al.* (2010) reported similar result

based on the macroscopic observation of lactic acid bacteria; white, entire and convex. The same results reported by Nur *et al.* (2017), that has a rounded, convex, elevation, flat edge, and a milky white color. Morphological characters lactic acid bacteria genus *Streptococcus* round or lenticular and white colonies (Sellami *et al.*, 2007). Meanwhile, a morphological macroscopic genus of *Lactococcus*, round or lenticular, white colonies, and very small and the genus of *Lactobacillus*, colonies in the maroon and convex center.



**Figure 1:** Colonies of bacteria on MRS agar and fungal colonies on PDA agar. (A) Isolates I<sub>1</sub> small size colonies; (B) Isolates I<sub>2</sub> medium size colony; (C) I<sub>3</sub> large colony size.

Results of microscopic examination are presented in Table 2. Bacterial isolate I<sub>1</sub> contained cocci- and bacilli-shaped bacteria and isolates I<sub>2</sub> and I<sub>3</sub> formed bacilli-shaped LAB. All three isolates are Gram-positive, non-spore forming and non-motile bacteria. Biochemical tests of the three races include catalase test and oxidase test shown to be negative, while positive results were obtained for aerobic and anaerobic testing. Based on macroscopic and microscopic observations, with addition of biochemical tests results, the three isolates were concluded as LAB which is facultative anaerobic. Identification of LAB from kefir grains shows all isolates are catalase-negative, rod-shaped or spherical. According to Axelsson *et al.* (2004), LAB is Gram-positive, rod-shaped or round, and catalase-negative. Battcock and Azam-Ali (1998) suggest that Gram-positive, spore-negative, cocci or bacilli-shaped, immobile (non-motile) and reacting adversely with hydrogen peroxide are all characteristics of the LAB. Rarah (1999) reported kefir and kefir microbes containing 35.4% *Lactobacilli* and 58.3% *Streptococcus*.

Based on Gram staining results, the morphology of the bacteria can be divided into two large groups, namely Gram-positive and Gram-negative bacteria. Gram-positive bacteria will appear purple in color, meanwhile, Gram-negative bacteria will appear pink (Hastowo, 1992). Results shown in Table 2 indicate all isolates are Gram-positive bacteria, based on the purple coloration.

Results of tests for temperature resistance indicate that all LAB isolates can grow at a temperature of 37 °C (100%); at 10 °C, growth was about 19.05%, and at 50 °C, there was no growth. One of the factors influencing bacterial growth is temperature. Chen *et al.* (2013) reported that bacterial growth based on temperature is grouped into three; (1) thermophiles, a minimum temperature range between 25-45 °C, (2) mesophyll (10-20 °C), and (3) psychrophiles (-5-0 °C). Wassie and Wassie (2016) reported physiological characteristics of resistance to temperature, where the genus *Streptococcus* can grow at 45 °C, but not at 10 °C; the *Lactococcus* genus can grow at 10 °C, 15 °C and 45 °C; and the genus *Lactobacillus* can grow at 15 °C and 45 °C, but not at 10 °C.

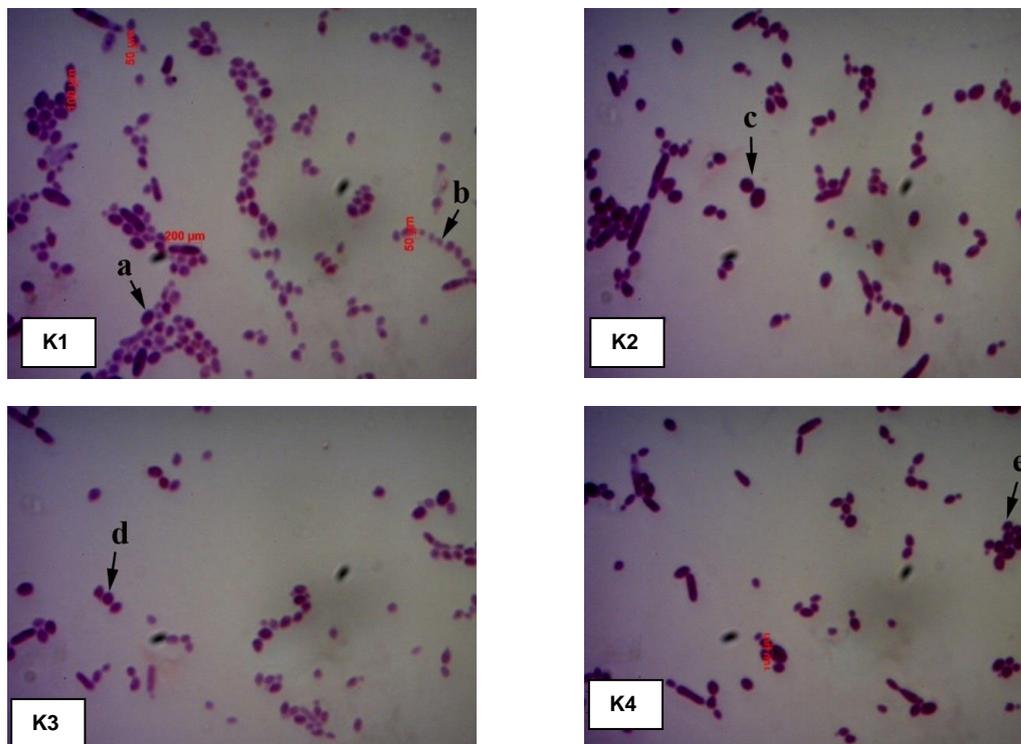
The catalase test determines the presence of the enzyme catalase in the bacterial starter culture. Bacteria that do not generate O<sub>2</sub> gas on H<sub>2</sub>O<sub>2</sub> drop test indicate that these bacteria have a peroxidase enzyme that can prevent bacterial O<sub>2</sub> and gas production and are described as a catalase-negative bacterium (Surono, 2004).

In the oxidase test, all isolates produced negative results, characterized by the absence of dark purple discoloration on the filter paper after addition of drops of *p*-dimethylamino aniline 1%. Motility test results, all non-motile isolates produced marked results in the absence of bacterial movement during the incubation period of 48 h indicated the absence of propagation at around pinprick inoculation loop needle. All isolates belonged to a LAB which does not have flagella.

**Table 2:** Microscopic observations of lactic acid bacteria isolated from Indonesian commercial kefir grains.

Isolates	code	Observation*			Growth temperature (°C)**			Motility test	Catalase test	Oxidase test	Aerob test	An aerob test
		Gram staining	Spore staining	Form	10	37	50					
I <sub>1</sub>	A1	+	-	Coccus	++	+++	-	-	-	-	√	√
	A2	+	-	Oval coccus	-	+++	-	-	-	-	√	√
	K1	+	-	Coccus	++	+++	-	-	-	-	√	√
	K2	+	-	Bacil, coccus	-	+++	-	-	-	-	√	√
I <sub>2</sub>	K3	+	-	Coccus	-	+++	-	-	-	-	√	√
	K4	+	-	Coccus	-	+++	-	-	-	-	√	√
	E1	+	-	Bacil	-	+++	-	-	-	-	√	√
	E2	+	-	Bacil	-	+++	-	-	-	-	√	√
I <sub>3</sub>	E3	+	-	Bacil	-	+++	-	-	-	-	√	√
	S1	+	-	Bacil	++	+++	-	-	-	-	√	√
	S2	+	-	Bacil	-	+++	-	-	-	-	√	√
	S3	+	-	Bacil	-	+++	-	-	-	-	√	√
	S4	+	-	Bacil	-	+++	-	-	-	-	√	√
	C1	+	-	Bacil	++	+++	-	-	-	-	√	√
	C2	+	-	Bacil	-	+++	-	-	-	-	√	√
	D2	+	-	Bacil	++	+++	-	-	-	-	√	√
D4	+	-	Bacil	-	+++	-	-	-	-	√	√	
I <sub>3</sub>	B1	+	-	Bacil	-	+++	-	-	-	-	√	√
	B2	+	-	Bacil	-	+++	-	-	-	-	√	√
	B3	+	-	Bacil	-	+++	-	-	-	-	√	√
	B4	+	-	Bacil	-	+++	-	-	-	-	√	√

\*+, positive Gram; -, negative; √, real found  
 \*\*+, growth; +++, strong growth; ++, weak growth; -, no grow



**Figure 2(a):** Observation of bacterial cell morphology for isolate I<sub>1</sub> using the Gram staining method magnification of 100× (a, *Lactococcus*; b, *Streptococcus*; c, *Streptococcus*; d, *Streptococcus*; e, *Lactococcus*).

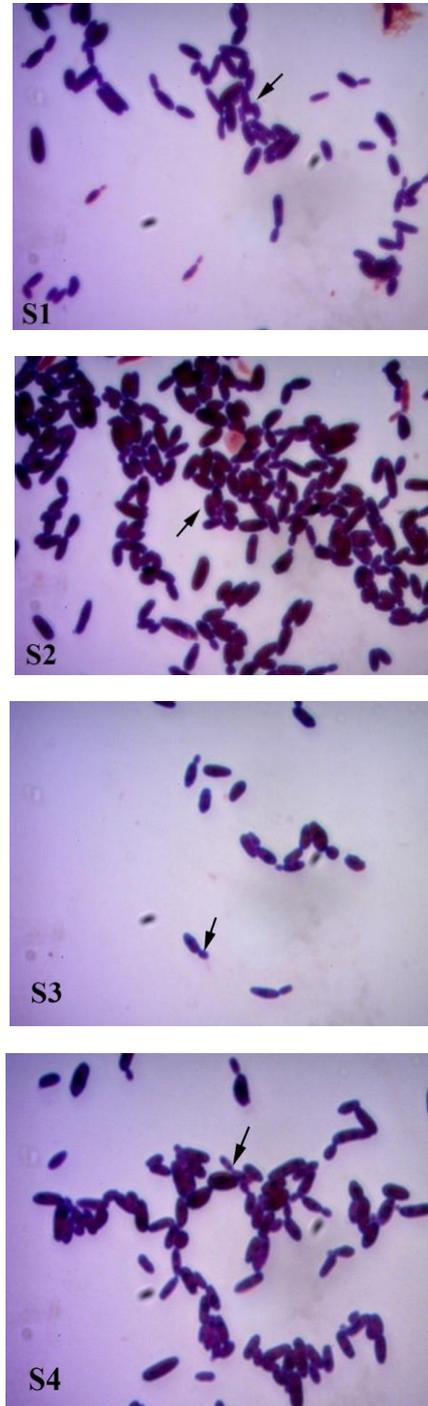
Figure 2a shows that the bacterial isolates (I<sub>1</sub>) include two types of the LAB: (a) shows LAB which is cocci-shaped and forms clusters; (b) shows LAB as chain-shaped cocci. Based on the results of the morphological examination, LAB was observed in (a) and (e) were considered as *Lactococcus* bacteria, while bacteria observed in (b), (c), and (d) had the appearance of *Streptococcus* bacteria. The results of morphological observation of *Streptococcus* bacteria by Vos *et al.* (2009) showed colonies of *Streptococcus* measuring 0.5-1.0 mm after incubation at 37 °C for 24 h and living in aerobic and anaerobic conditions. Further, according to Cowan and Steel (2003), *Streptococcus* bacteria have characteristics which include Gram-positive, cocci-shaped and non-motile. According to Sneath *et al.* (1986), the morphology of microscopic round or ovoid, in pairs or form long chains. Andayani *et al.* (2008) described *Lactococcus* bacteria with morphological features as follows: colonies of milky-white or slightly creamy color, forming spherical or large colonies, spherical cells which has diameter of 0.5-1.2 × 0.5-1.5 µm, in pairs and form short chains in liquid media, non-spores, Gram-positive and non-motile, catalase-negative and oxidase-negative.

In a study by Wassie and Wassie (2016), LABs isolated from fresh milk were Gram-positive, catalase-negative and non-motile. The LABs contained *Lactobacillus* (26.51%), *Lactococcus* (21.69%), *Leuconostocs* (18.07%), *Streptococcus* (9.64%), *Pediococcus* (12.05%) and *Enterococcus* (9.64%). The difference between *Streptococcus* and *Lactococcus* is based on the shape, where *Streptococcus* are cocci and chain-shaped while *Lactococcus* are cocci and round-shaped.

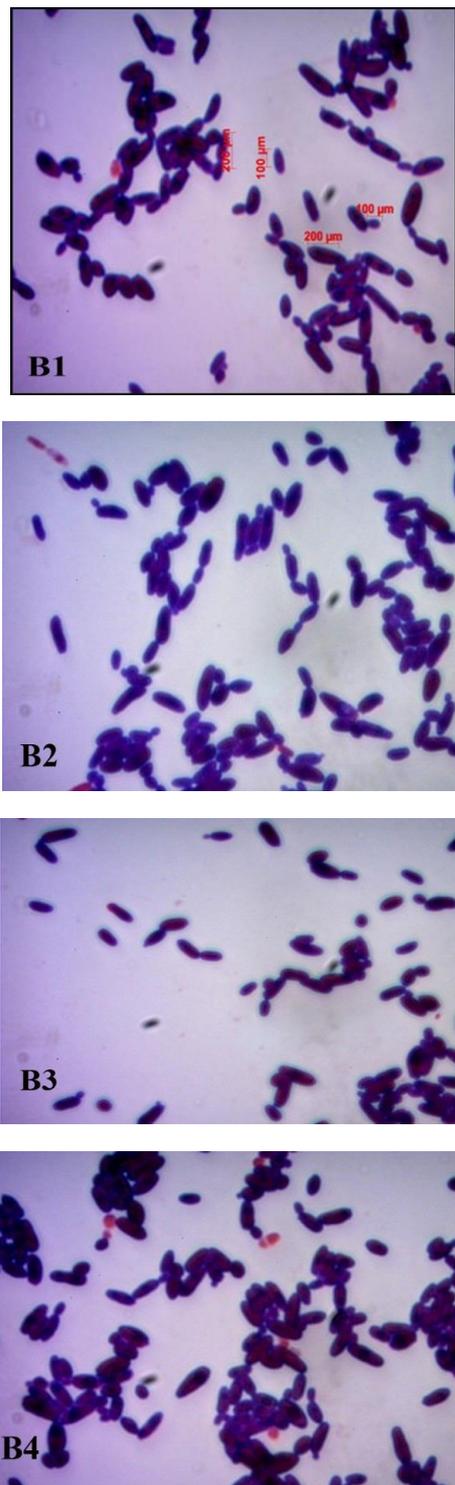
Figure 2b and 2c shows the bacteria morphological forms, which appear to have the same shape. The results of a morphological observation made in accordance with the description including *Lactobacillus*. Sameen *et al.* (2010) reported that *Lactobacillus* incubated for 48 h at 37 °C in MRS medium can live in aerobic and anaerobic conditions, has a colony size of 1.5-2.0 mm, is convex and entire. *L. plantarum* bacteria are Gram-positive, rod-shaped bacteria, found in pairs or chains. *L. plantarum* is the largest among the species of the LAB (Buckle *et al.*, 1987). Further, Kuswanto and Sudarmadji (1988) reported that *L. plantarum* grown in agar medium can form colonies which diameter were about 2-3 mm, are opaque white in color and convex shaped, and the known as lactic acid-forming bacteria.

*Lactobacillus plantarum* is one of LAB homofermentative with optimal temperatures below 37 °C (Frazier and Westhoff, 1978). *L. plantarum* rods (0.5-1.5 until 1.0-10 mm) and non-motile. The bacteria are catalase-negative, aerobic or facultative anaerobes, able to dissolve gelatin, fast digesting protein, does not reduce nitrate, acid tolerant and capable of producing lactic acid. According to Goldin (1998), *L. plantarum* is one LAB that is important in the fermentation of meat, milk, and vegetables. *L. plantarum* is a LAB of the Lactobacillaceae family, genus *Lactobacillus* and subgenus *Streptobacterium* (Pelczar *et al.*, 1983). According to Ray

(2004), *L. plantarum* bacteria are Gram-positive, rod-shaped, single or short-chained, non-spore, catalase-negative and facultative anaerobic bacteria.



**Figure 2(b):** Observation of bacterial cell morphology for isolate I<sub>2</sub> using the Gram staining method magnification of 100 × (Arrow: *Lactobacillus*).



**Figure 2(c):** Observation of bacterial cell morphology for isolate I<sub>3</sub> using the Gram staining method magnification of 100 × (Note: *Lactobacillus*).

Based on morphological observations, the type of bacteria and the type of early cultures used are cultured kefir grain powder contained bacteria *Lactococcus cremoris*; *Streptococcus cremoris* and *Lactobacillus plantarum*. The results of morphological examination also demonstrated the presence of *Lactobacillus plantarum*, *Lactococcus cremoris* and *Streptococcus cremoris* bacteria.

## CONCLUSION

Lactic acid bacteria isolated from kefir grains were identified by macroscopic and microscopic examination of cell morphology and using biochemical tests were putatively identified as *Lactococcus cremoris*, *Streptococcus cremoris* and *Lactobacillus plantarum*. Identification of the bacteria genus was based on taxonomy conventional method and supported with macroscopic, microscopic and biochemical characteristic observation. Then the result of observation was compared with text book to obtain the genus of bacteria.

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