Biosaintifika 11 (1) (2019) 39-46

Biosaintifika
Journal of Biology & Biology Education

http://journal.unnes.ac.id/nju/index.php/biosaintifika

Selection of Tempoyak Lactic Acid Bacteria As Candidate Strain for Yoghurt Starter Culture

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DOI: http://dx.doi.org/10.15294/biosaintifika.v11i1.16769

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History Article

Received 16 November 2018
Approved 19 January 2019
Published 30 April 2019

Keywords

Lactic acid bacteria; Tempoyak; Yoghurt

Abstract

Selection of bacteria in yoghurt fermentation is important to produce yoghurt with good quality. *Tempoyak* lactic acid bacteria is potential to be yoghurt starter culture becouse *tempoyak* fermentation has similarities in producing lactic acid such as yoghurt. This study aimed to isolate and identify the lactic acid bacteria (LAB) from tempoyak which will be used as a yoghurt starter culture. The methods used in this study included isolation and selection of acid-producing bacteria, lactase and protease activity test, identification of morphology and biochemistry as well as testing the quality of the yoghurt. The results of the study obtained 32 isolates of the LAB with the same characteristic colony, include the round shape, cream-coloured with convex elevation and, smooth surface and entire edge. Selection of acid-producing bacteria obtained 12 isolates with the ability to produce clear zones on MRSA + CaCO3 media ≥ 0.7 cm. Selection of lactase-producing LAB obtained six strains and the protease test obtained two superior strains. Two superior strains namely Tp 12 and Tp 28 have characteristics of coccus, gram-positive, negative catalase, non-endospore and non-motile forms. The organoleptic and several quality tests showed yoghurt using Tp 12 as starter has higher acceptability, the highest levels of lactic acid and lactose levels with values respectively 4.25, 0.84% and 24.53%. This study obtained the LAB strain which can be used as yoghurt starter culture. Tp 12 strain can be used to improve the quality of yoghurt and become a commercial starter that can be applied to various fermented products.

How to Cite

Rahayu, H. M., & Qurbaniah, M. (2019). Selection of Tempoyak Lactic Acid Bacteria As Candidate Strain for Yoghurt Starter Culture. *Biosaintifika: Journal of Biology & Biology Education*, 11(1), 39-46.

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p-ISSN 2085-191X
e-ISSN 2338-7610

INTRODUCTION

Yoghurt is one of the processed drinks made through the fermentation process and have a sour taste (Harjiyanti et al., 2013). Yoghurt fermentation process involves Lactic Acid Bacteria (LAB) who will carry out the fermentation process on carbohydrates and the production of lactic acid as the main product of fermentation. The primary use of LAB is as a starter culture for various fermented milk products including cheese and yoghurt (Bintsis, 2018).

Starter culture has an important role in the process of making yoghurt because it will determine the quality of the yoghurt produced. For this reason, it is necessary to choose the right starter culture so that the yoghurt produced has good quality. Starter bacteria that are often used in the process of making yogurt consist of a mix of *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. Bulgaricus* (Aswal, et al., 2012). The use of lactic acid bacteria strains from local products as a starter allows to improve the quality of yoghurt. One of the local products that is potential to be the starter on Yoghurt production is Lactic Acid Bacteria (LAB) from tempoyak.

Tempoyak is food from Indonesia made from fermented durian meat (*Durio zibethinus* Murray) (Widowati et al., 2013). During the fermentation process, the microorganisms that play a role are dominated by Lactic Acid Bacteria (LAB) (Leisner et al., 2002)aMalaysian acid-fermented condiment. In a study on the diversity of LAB in thisproduct, three isolates could not be identified using SDS-PAGE of whole-cellproteins or API 50 CH. The taxonomic position of the three isolates wasclarified in the present study. 16S rDNA sequencing classified a representativestrain in the genus Lactobacillus, clearly separated from all known species, andmost closely related to the Lactobacillus reuteri phylogenetic group. DNA–DNAhybridization experiments and an extensive phenotypic description confirmthat the strains represent a single and separate novel species among theobligately heterofermentative lactobacilli. The three isolates are distinguishedat the intra-species level by plasmid profiling, pulsed-field gel electrophoresisof macro-restriction fragments and biochemical features. The nameLactobacillus durianis sp. nov. is proposed for the novel taxon and the typestrain is LMG 19193T(l CCUG 45405T; (Chuah et al., 2016). The LAB is a microorganism that is widely distributed in various environments that are rich in carbohydrates such as plants, fermented foods, human mucosal surfaces and in land and sea animals (Florou-Paneri et al., 2013). Especially in *tempoyak,* LAB can grow by fermenting simple sugars from durian meat and turning them into lactic acid and acetic acid (Widowati et al., 2013).

Some previous researches showed that lactic acid bacteria is microorganism that play dominant role during fermentation process (Amiza, et al., 2006). The study of the use of LAB tempoyak isolates as yoghurt starter has never been done before. The objective of this study was to obtain LAB isolate from *tempoyak* that can be used as yoghurt starters. The results of this study was expected to improve the quality of yoghurt and can be used as a commercial starter for various fermented products.

METHODS

Ripe durian fruit was peeled and separated from seeds. Then about 2% salt (b/b) was added to seedless durian meat and they were put in a tightly closed jar created an anaerobic atmosphere. Jars were left at room temperature for one week. After one week, the jar were opened, and the *tempoyak* was ready to be isolated.

Lactic Acid Bacteria Isolation

One grams sample was added into 9 ml sterile distilled water and homogenized with the vortex. Serial dilution was performed until the concentration reach 10^{-7}. One ml of the homogenized and diluted samples were poured into Petri dishes. This culture was incubated on temperature of 30 °C for 48 hours. Culture was then purified on MRS Media and was incubated on temperature of 30 °C for 48 hours. Purified culture was stored in MRS agar slant on temperature of 4-10 °C.

Selection of Acid-producing LAB

In order to select the Lactic Acid Bacteria (LAB) that have potential on producing acid, selection process using MRS Media + CaCO3 0.5% was conducted. Incubation was done for 68 hours at room temperature (26°C). The clear zone around the bacteria colony showed that those bacteria produced acid. Clear zone measurement was done by determining the bacteria ability on producing acid with clear zone index formula:

$$\text{Acid clear zone index} : \frac{\text{acid clear zone diameter}}{\text{colony diameter}}$$

Selection of LAB Producing Protease and Lactase

Determination of protease producer LAB was done by using SMA (Skim Milk Agar) medium and milk pasteurization. Incubation was done for 68 hours at room temperature (26°C). While, the determination of lactase producer LAB was conducted using TSIA (Triple Sugar Iron Agar) media with the incubation at temperature of 37°C for 24 hours. The culture positively formed protease and lactase marking with the formation of clear zones. Measurement of the clear zone area was by the formula:

$$\text{Clear zone index} = \frac{\text{(clear zone diameter)}}{\text{colony diameter}}$$

Identification of Lactic Acid Bacteria

The superior isolates obtained were then identified by several tests included gram staining, endospore staining, catalase and, motility testing.

Starter Making Process

UHT milk was prepared and pasteurize at 60-65 °C for 30 minutes. The milk temperature was lowered to 45 °C and inoculated with LAB from tempoyak. Then was incubated at 25-27 °C (room temperature) for 24 hours.

Yoghurt Making Process

100 ml UHT milk pasteurized at 61-65 °C for 30 minutes. Then, 5 grams of skim milk and, 4 grams of sucrose were added. The temperature was then lowered to reach 45 °C, and 5 ml of the yoghurt starter was added, and it was stored at room temperature for 12 hours.

Organoleptic Test

Organoleptic tests (preference test) was conducted on 15 panelists who were students of Biology Education of University of Muhammadiyah Pontianak. Organoleptic assessment was done by giving the samples of yoghurts to the panelists and asking the them to taste each sample given. Then, each panelist filled in an assessment form consisting of assessment of flavor, aroma, thickness, and color. The range of scores given was 1-5 with the description as follow: 1 (dislike very much), score 2 (dislike), score 3 (like slightly), score 4 (like), score 5 (like very much).

pH Measurement

The pH measurement was performed using a pH meter. The pH meter was set with a buffer solution to a value of 7, then the pH meter dipped into the yoghurt to read its pH value.

Measurement of Lactic Acid Level

Acidity values were calculated using the Mann acid test method. The sample was put into 10 ml of Erlenmeyer and added with five drops of 1% phenolphthalein indicator then it was titrated using a 0.1 N NaOH solution until the color turned to pink. The amount of 0.1 N NaOH solution needed for the titration can be determined with the following formula:

$$\% \text{ acidity} = \frac{\text{(ml NaOH} \times 0.009 \times 100\%)}{\text{volume sample}}$$

Measurement of Lactosa Levels

About 25 ml sample was put into a 50 ml volumetric flask, and 5 ml ZnSO reagent was added and homogenized. Then, 5 ml NaOH 0.75 N was added and homogenized. Dilution was performed using distilled water until the marking line on the measuring flask. Let the solution stand for ± 10 minutes to get all the protein. Then, it was filtered with filter paper and the filtrate was collected. The filtrate volume was calculated theoretically using the volume of protein deposited from the initial volume. About 5 ml of filtrate was put into erlenmeyer and added with 20 ml aquadest, 20 ml of KI 10% and, chloramine-T as much as 50 ml. Erlenmeyer was locked and the solution was homogenized, then let it stand for 90 minutes. As much as 10 ml of 2N HCl was added to the solution then was titrated with 0.1N Na-2S2O3 until a stable, pale yellow colour was obtained. Starch solution was then added and then titration was performed until the color turned to grey.

Lactose content was calculated by formula:

$$A=(Tb-Ts)\times N\times 0.171 \ \frac{100}{5}$$

Then the lactose content in 100 ml of milk was calculated with the following formula:

$$\text{lactose content of 100 ml milk} = A \times \frac{48.4}{100} \times \frac{100}{5}$$

A = g lactose / 100 ml filtrate
Ts = sample titration
Tb = blank titration
N = normality of Na2S2O3

RESULTS AND DISCUSSION

Acid-Producing Lactic Acid Bacteria (LAB) Selection

Isolation of acid-producing LAB was carried out on MRSA media with the addition of CaCO3. The isolation results obtained 32 colonies with the same characteristics of colony morphology included round colonies, cream-coloured with convex elevation, smoot surface and entire edges (Figure 1). The characteristics of the colony's morphology showed the similarity with the characteristics of lactic acid bacteria in general (Pundir et al., 2013).

Isolates that have the acid-producing ability are characterized by the formation of clear zones around the colonies in the media (Figure 1). The clear zone shows that LAB can use glucose as an energy source and producing the secondary metabolites in the form of acid. Acidic compounds from LAB can degrade CaCO3 to Ca lactate which indicates by the formation of clear zones around the LAB colony (Nuryady 2014).

Lactase-Producing Lactic Acid Bacteria (LAB) Selection

The next stage of the research was screening 15 isolates of selected LAB in TSIA media to see the ability of these isolates to produce lactase enzyme. Lactase or β-Galactosidase is an enzyme known as the biocatalyst to hydrolyze lactose in milk into glucose and galactose (Abd El-Kader, et al., 2012). Lactase is widely used to prevent the process of lactose crystallization, increase the level of sweetness, and increase the solubility of milk products (Princely et al., 2013)

Screening results of 15 LAB from tempoyak isolate on TSIA media showed that 14 isolates could produce lactase indicated by the formation of clear zones around the colony. The results of the measurement of the clear zone of the fourteen strains obtained six isolates with clear zone diameter > 1.5 cm (Figure 2).

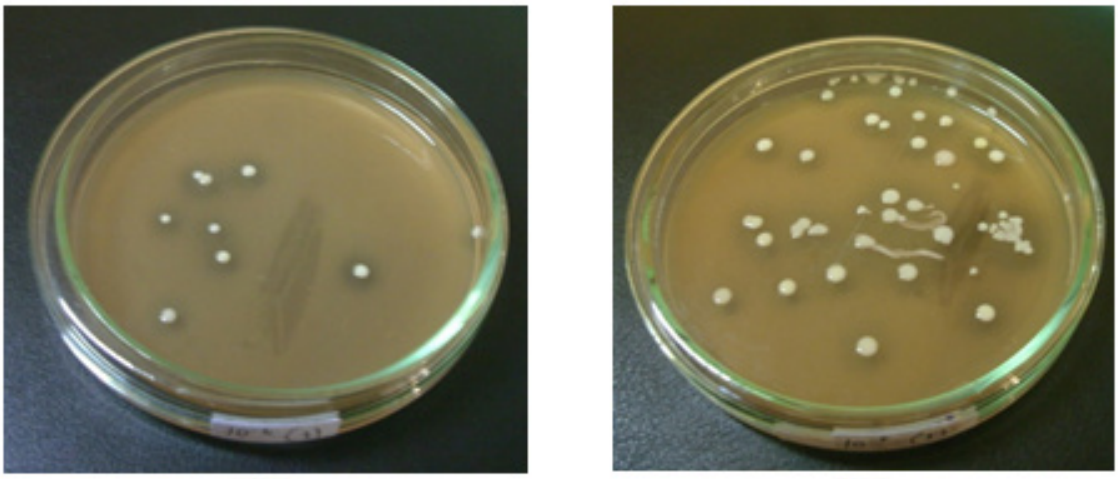
Protease-Producing Lactic Acid Bacteria (LAB) Selection

Protease is an enzyme that catalyses the hydrolysis process of protein peptide bonds (Phyu et al., 2015) and breaks down polypeptides into amino acids (Alnahdi, 2012). Identification of protease-producing LAB was done by growing the bacteria in skim milk agar media. Bacterial proteolytic activity was characterized by the formation of clear zones around the isolates (Dajanta et al., 2009; Tennalli et al., 2012; Phyu et al., 2015; Hamza, 2018). Result of protease test showed that six LAB isolates showed a positive proteolytic activity characterised by the formation of clear zones around the colonies. The clear zone correlates with the amount of protease produced by the bacteria. Proteolytic activity is one of the important characteristics of lactic acid bacteria (Phyu et al., 2015). The results of the measurement of clear zone diameter obtained two superior isolates namely Tp 12 and Tp 28 with 3.15 cm and 4 cm clear zone diameters respectively (Figure 3).

Gram Staining

Gram staining is used to distinguish gram-positive and gram-negative bacteria. The purple colour of gram-positive bacteria appears because gram-positive bacterial cells have a thicker peptidoglycan structure and an inner membrane. The peptidoglycan will bind the colour of crystal violet so that the colour will not fade after the addition of ethanol. In the end, the colour of gram-positive bacterial cells will remain purple (Madigan et al., 2012).

The results of gram staining showed that



(a) (b)

Figure 1. Acid-producing LAB in MRSA + CaCO3 dilution series (a) 10-6 dan (b) 10-5

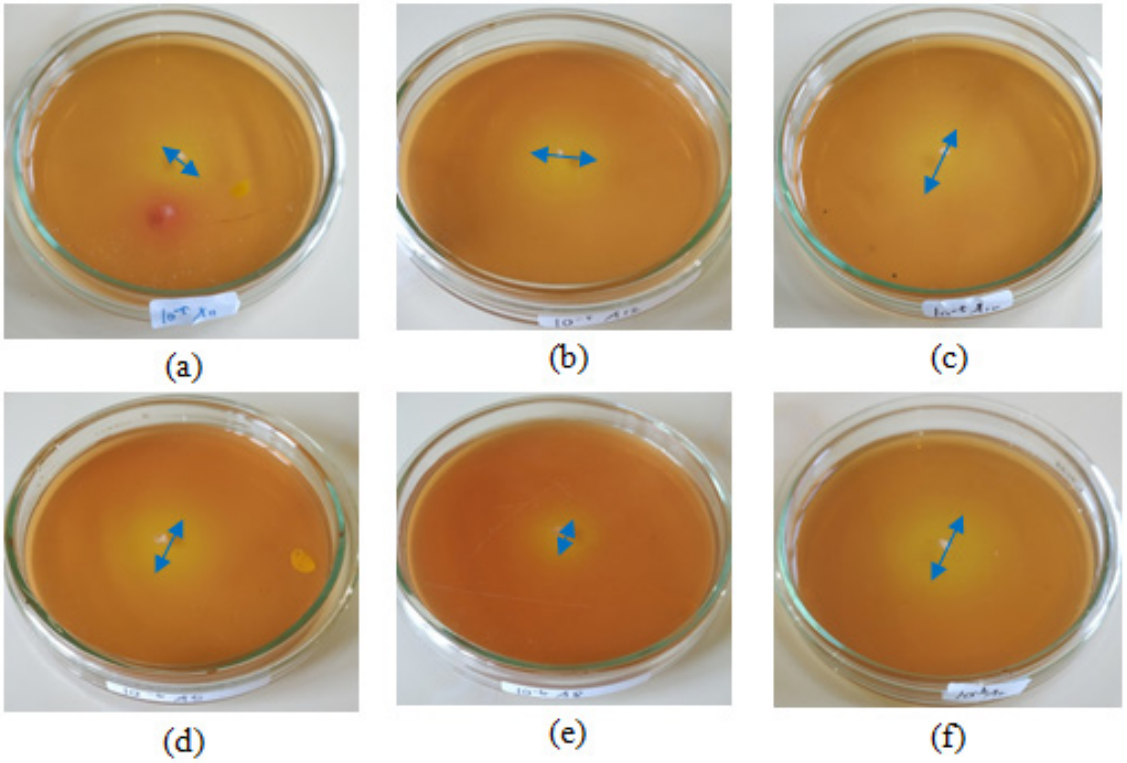


Figure 2. Clear zone on TSIA (a) Tp 11; (b) Tp 12; (c) Tp 10; (d) Tp 28; (e) Tp 31; (f) Tp 25

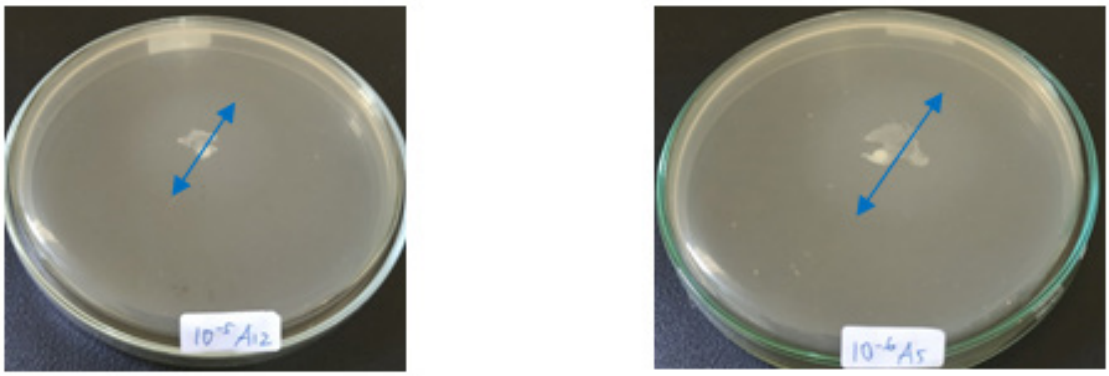


Figure 3. Clear zone formed on Skim Milk Agar (a) Tp12 dan (b) Tp 28

two superior LAB isolates, Tp 12 and Tp 28, were gram-positive bacteria (Figure 4). This indicates that both strains have a thick peptidoglycan layer on the cell wall structure (Hamza, 2018). Gram-positive is an absolute property that must be possessed by the LAB. This is in line with the research by Leisner et al. (2002); Neti & Dizon, (2011); S. Widowati & Miagiyarta, (2007); (Mahulette, Mubarik, Suwanto, & Widanarni, et al. (2018) which showed that the nature of LAB isolated from tempoyak have gram-positive properties. The shape of isolate cells is coccus. Based on the shape and result of gram staining, it is suspected that these two isolates are *Streptococcus* sp.

Endospore Staining

In addition to gram staining, endospores staining was also carried out in this study. The test results showed that two isolates (Tp 12 and Tp 28) reacted negatively to endospore staining (Figure 5). Based on the results of the identification, it can be concluded that these two isolates are LAB.

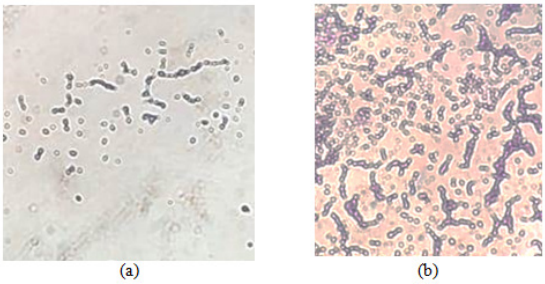


Figure 4. Morphology of (a) Tp 12 and (b) Tp 28 showed gram-positive

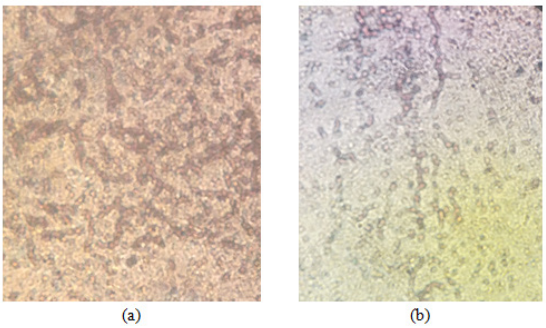


Figure 5. Endospore staining of isolates (a) Tp 2 and (b) Tp 28 which show non-endospores result

Catalase Test

Catalase test was carried out to identify microbes capability in producing catalase enzymes. Catalase enzyme is used to break down hydrogen peroxide formed during aerobic respiration that is toxic to bacteria to become non-toxic dihydrogen oxide and oxygen (Mardalena, 2016). Tp 12 and Tp 28 catalase tests showed negative results (Figure 6) because isolates dripped with hydrogen peroxide did not produce any bubbles. One characteristic of LAB is catalase-negative (Neti & Dizon, 2011); (Pundir et al., 2013); (Widowati et al., 2013); (Chuah et al., 2016); (Khalil & Nural, 2016)pasteurized milk, locally and commercially manufactured yoghurts samples were evaluated for total viable count of Lactic Acid Bacteria (LAB. Thus, it can be said that these two isolates are a LAB.

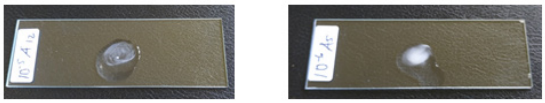


Figure 6. Catalase test results of isolate (a) Tp12 and (b) Tp 28 show a negative reaction

Motility Test

Motility test is also one test that can be used to distinguish between LAB and other bacteria. In motility testing, two isolates were subcultured on semi-solid upright MRS media and incubated for 48 hours at 30 ^{0}C. Results of this test showed non-motile results because the strains only grew along the puncture marks (Figure 7).

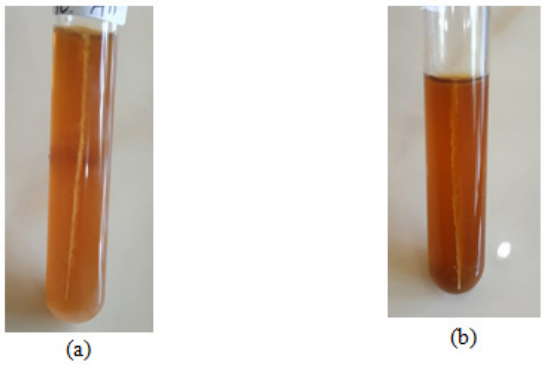


Figure 7. Motility test of isolate (a) TP 12 and (b) Tp 28 show non-motil result

Organoleptic Test

The measurement of the level of people's preference for yoghurt using Tp 12 and Tp 28 as starter was carried out on 15 panelists using an organoleptic test questionnaire. Organoleptic test questionnaires include preferences for the colour, aroma, taste and thickness of yoghurt. Testing the level of preference using a scale of one to four (1-5) with the criteria as follow: one (dislike very much), two (dislike), three (light slightly), four (like), and five (like very much). The results of the questionnaire calculations are presented in the following table:

Table 1. Organoleptic test of yoghurt using Tp 12 & Tp 28

Aspect	Tp 12	Tp 28
Colour 4.53		4.27
Smell	4.33	2.4
Taste	4	1.6
Thickness	4.13	4
Average	4.25	3.06

Based on organoleptic test, yoghurt Tp 12 higher than yoghurt Tp 28 (Table 1). Yoghurt Tp 12 has sweet and sour taste and the aroma of fermented milk, while yoghurt using TP 28 has bitter flavour and musty smell. So that, people prefer yoghurt with Tp 12.

Yoghurt Quality

Quality of yoghurts using Tp 12 and Tp 28 starter were determined by the measurement of pH, lactic acid and lactose content with the following results:

Table 2. Quality yoghurt using TP 12 & Tp 28 starter

Isolate	pH	Lactic acid content (%)	Lactose content (%)
Tp 12	5.6	0.84	24.53
Tp 28	5.3	0.582	18.5

The results of pH measurements of both yoghurt samples obtained values of 5.6 and 5.3. The decrease in pH occurs because lactic acid bacteria produce lactic acid during the lactose fermentation process in milk takes place (Eke et al., 2013). The streptococci is responsible for the initial pH drop of the yogurt mix to approximately 5.0. The lactobacillus are responsible for a further decrease to pH 4.5 (Aswal et al., 2012). The highest levels of lactic acid and lactose levels was found in yoghurt using Tp 12 as a starter with values respectively 0.84% and 24.53%.

Based on the organoleptic and several quality tests, yoghurt using Tp 12 was the best quality becouse it has higher acceptability, the highest lactic acid and lactoses content. So, Tp 12 can be used as a starter culture in the process of yoghurt fermentation. These strain also can be used to improve the quality of yoghurt and become a commercial starter that can be applied to various fermented products.

CONCLUSION

One isolate of LAB tempoyak namely Tp 12 can be starter of yoghurt fermentation. Organoleptic and several quality tests showed yoghurt using Tp 12 as starter has higher acceptability, the highest levels of lactic acid and lactose levels with values respectively 4.25, 0.84% and 24.53%.

ACKNOWLEDGEMENT

Author thank to Ministry of Research, Technology and Higher Education for the financial support in Beginner Lecturer Research Scheme on 2018.

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