ORIGINAL ARTICLE

Biochemical characterization of lactic acid bacteria (LAB) isolated from home-made fermented durian flesh, *tempoyak*

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Abstract:

Tempoyak is a fermented condiment made from durian fruit (*Durio zibethinus*). *Tempoyak* is prepared by the separation of flesh durian pulp from seed and then the flesh durian pulp is left to ferment for 7 days at ordinary temperature. Enumeration of lactic acid bacteria (LAB) is done on MRS agar and isolated single colony is picked up to perform morphological, cultural and biochemical test. The isolated colony from *tempoyak* showed LAB properties that include the ability to grow in MRS agar, Gram positive bacteria as well as negative for catalase test. Out of 10 from 28 bacteria that were isolated from *tempoyak* were categorized as LAB base on their morphological, cultural and biochemical characteristics. Identification of LAB is confirmed by using API 50 CHL strips kit. The results of API 50 CHL revealed 3 out of 10 are *Lactobacillus plantarum* and it is the predominant species in *tempoyak.* The other species that have been recognized were *Lactobacillus brevis, Lactobacillus pentosus, Lactobacillus fermentum, Lactobacillus lactis, Lactobacillus raffinolactis, Lactobacillus casei,* and *Leuconostoc mesenteroides.* Meanwhile, the LAB count varied in the range between 5.3×10^{10} to 1.24×10^{12} cfu/ml.

Keywords: Colony count, lactic acid bacteria, pH, *tempoyak*

1. INTRODUCTION

Tempoyak is a fermented condiment made from meat of durian (*Durio zibethinus*). It is famous in Malaysia and Indonesia as a dish and condiment because of its distinctive durian smell and creamy yellow color [1-3]. Currently, research on *tempoyak* is very limited as previous study was more focused to contributing new knowledge which are valuable in producing standard commercial *tempoyak* which is uniform and of high quality [4].

Tempoyak is prepared with or without salt which supports growth of lactic acid bacteria (LAB) [5]. Generally, species of *Lactobacillus* sp., *Leuconostoc* sp., and *Streptococcus* sp. grow in *tempoyak* [6] with *Lactobacillus plantarum* are the predominant group of LAB in *tempoyak* [7]. This evidence is strongly agreed by Yuliana and Dizon, [8] which identified *L. plantarum* as the main LAB isolated from *tempoyak* made in Malaysia and Indonesia. Other than *L. plantarum,* species of *Lactobacillus brevis, Lactobacillus mali* and *Lactobacillus fermentum* were also isolated from Malaysian *tempoyak* [9], whereas *L. plantarum, Lactobacillus casei, Lactobacillus corynebacterium* were the common species of LAB isolated from Indonesian *tempoyak* [10]. Other species found were *Leuconostoc durianis* from Malaysian *tempoyak* [11], *Leuconostoc mesenteroides*, *Pediococcus acidilactici, Weseilla mesenteroides* [12].

LAB have beneficial properties and acts as probiotics to maintain humans and animals healthy diet. In recent years, different studies also support the importance of probiotics as a way to provide a natural, safe and effective barrier against microbial infections [13, 14]. According to the definition by the World Health Organization (WHO), probiotics are "live microbial food supplements which, when administered in adequate amounts confer a health benefit on the host" [15]. Study done by Collins and Gibson [16] stated that, among the usually used microorganisms, LAB are regarded as a major group of probiotic bacteria. They are non-pathogenic, acid and bile tolerance and also produce antimicrobial substances and technologically suitable for industrial processes [17]. They are classified as safe (GRAS) microorganisms because of their long and safe use as starter cultures in fermented products [18], thus the aim in this present study is to isolate and identify the LAB in homemade *tempoyak* for the potential use in development of probiotic in new fermented food and other industries.

2. MATERIALS AND METHODS

2.1 Preparation of homemade *tempoyak*

Durian was bought from local market in Shah Alam, Selangor, Malaysia. Flesh durian pulps were separated from seed, stored in sealed container and left to ferment at ordinary temperature (28-34ºC) for 8 days. The preparation of homemade *tempoyak* was done in the Microbiology laboratory in UiTM Puncak Alam Campus.

2.2 Isolation of LAB

After 8 days of the fermentation of *tempoyak*, 25 g of the homemade *tempoyak* were added to 225 ml of 0.1% (w/v) sterile peptone water and another 25 g were added to 225 ml of Man Rogosa Sharpe (MRS) broth enriched with sucrose [19]. Both *tempoyak* were left to homogenize in shaking incubator for 5 days at 37ºC.

2.3 Enumeration of LAB

After *tempoyak* were homogenize for 5 days at 37ºC, serial dilution was performed up to 10^{-8}. A 100 ul of 10^{-8} serial diluted *tempoyak* were aseptically transferred to MRS agar. Then the samples were spread onto MRS agar by using spreader. The plates were incubating in anaerobic jar at 37ºC for 3 days. After incubation, the colonies were randomly selected and picked up to be subcultured on MRS agar for several times until pure colonies were obtained. The pure colonies were maintained in MRS agar slant and subcultured on MRS broth as stock culture and stored at 4ºC for storage [20].

2.4 Identification of lactic acid bacteria

The cultures were identified by their morphological, cultural and biochemical test. Bacteria were examined by the following test; Gram reaction, catalase production, production of oxidase, test for motility which was done in SIM broth, hydrogen peroxide production, Hugh and Leifson's oxidative/ fermentation [21], reaction fermentation of sugar (arabinose, D-mannitol, galactose, raffinose, glucose, lactose, maltose, sucrose, sorbitol, fructose, cellobiose, trehalose, salicin, esculin, ramnose, mellobiose, mannose, xylose and melezitose), indole production in trypthone broth, growth at 2%, 4% and 6.5% in NaCl and growth at pH 4.5, 6.5 and 7. The confirmation of species was done by using API 50 CH in conjunction with API 50 CHL strip kit [22].

2.5 LAB count

Twenty-five g of *tempoyak* was mixed with 225 ml sterile 0.85% (w/v) saline water in duplicate. The mixture was shaken to distribute the organism uniformly. Serial dilution was carried out until the dilution factor of 10^{-8}. Sample of 0.1 ml was pipetted and spread on MRS agar. Incubation was carried out under anaerobic condition at room temperature for 7 days. Plates containing 25-250 colonies were counted [20].

2.6 Determination of pH

About 10 to 20 ml of distilled water was taken and poured into a beaker. Temperature of the prepared paste was adjusted to 25ºC. The electrodes were immersed in the sample and the pH reading was taken after allowing the meter to stabilize. The pH readings were documented for 7 days [20].

3. RESULTS

3.1 Isolation and identification of LAB from homemade tempoyak

Out of the 28 bacteria that were isolated from the prepared homemade *tempoyak*, 10 were identified as LAB. All of the LAB isolated were classified based on their morphology, biochemical characteristic and other characteristic by tested with several tests. All isolated species were Gram positive, catalase negative, non-motile, non-spore forming and Indole negative but they have different reactions in oxidative fermentation and biochemical sugar tests. Although they were Gram positive bacteria but their shape varied between species. Based on the shape, arrangement and cell form which were observed under light microscope, the bacteria were either cocci, rod or short bacilli. In addition, the supplement tests such as growth in different pH and different concentrations of sodium chloride (NaCl) as well as growth at 15ºC and 45ºC also were carried out. Result of physiological and biochemical characteristic of isolated strain (LAB) were presented in Table 1.

Table 1: Physiological and biochemical characteristics of isolated strains (LAB).

Biochemical test	**LAB 1**	**LAB 2**	**LAB 3**	**LAB 4**	**LAB 5**	**LAB 6**	**LAB 7**	**LAB 8**	**LAB 9**	**LAB 10**
Gram stain	+	+	+	+	+	+	+	+	+	+
Cell shape	Rod	Rod	Thin rod	Rod	Rod	Cocci	Rod	Rod	Rod	Short rod
Other test:										
Catalase	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-
SIM	- /-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
MR	+	+	+	+	+	+	+	+	+	+
Oxidative/	-	+	-	-	+	+	+	+	-	-
Fermentation	+	+	+	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	-	-	-	-
Growth in:										
2% NaCl	+	-	+	-	-	-	-	+	+	-
4% NaCl	+	+	+	-	-	-	-	+	+	-
6.5% NaCl	-	-	-	-	-	-	-	-	-	-
Growth in:										
pH 4.5	+	+	+	+	+	+	+	+	+	+
pH 6.5	+	+	+	+	+	+	+	+	+	+
pH 7	+	+	+	+	+	+	+	+	+	+
Growth at:										
15ºC	+	+	+	+	+	+	+	+	+	+
45ºC	-	-	-	-	-	-	-	-	-	-
Sugar set:										
Arabinose	-	-	-	-	-	-	-	-	-	-
D-mannitol	+	+	+	-	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	-	-	+	-	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	-	+	+	+	+
Maltose	+	+	+	+	+	+	+	-	+	+
Sucrose	+	+	+	+	+	+	+	-	+	+
Sorbitol	+	-	+	-	+	-	-	-	+	-
Fructose	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-	-	-	-
Mellobiose	+	+	+	+	+	-	+	-	+	+
Melizitose	+	-	+	-	+	-	+	+	+	-
Mannose	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	-	+	+	+	+	-	+	+
Identified by API strips as	L.pt1	L.br1	L.pt1	L.fr2	L.pe	L.lac1	L.rf	L.ca	L.pt1	L.mo
Identification probabilities (%)	96.8	99.9	99.9	99.9	97.9	99.9	99.9	99.7	98.4	97.7

+ positive reaction; - negative reaction

L.pt1 : *Lactobacillus plantarum 1*
L.br1 : *Lactobacillus brevis 1*
L.fr2 : *Lactobacillus fermentum 2*
L.pe : *Lactobacillus pentosus*
L.lac1 : *Lactococcus lactis 1*
L.rf : *Lactobacillus raffinolactis*
L.ca : *Lactobacillus casei*
L.mo : *Leuconostoc mesenteroides*

3.2 Determination of LAB count and pH

The colony count of LAB and the pH reading are presented in Table 2.

Table 2: Colony count and pH reading.

Days	Colony count (cfu/ml)	pH reading
0	0	6.65
1	1.24×10^{12}	3.85
2	1.23×10^{12}	3.87
3	7.33×10^{10}	3.93
4	5.33×10^{10}	4.01
5	3.2×10^{11}	4.08
6	3.33×10^{11}	4.13

4. DISCUSSION

LAB are classified into species based on their morphology, reaction in biochemical tests and other psychological characteristic. Out of the 28 bacterial samples isolated, 10 bacteria revealed characteristics of LAB such as Gram positive, catalase negative, non-motile, non-spore forming and indole negative. Similar pattern of results was obtained for all samples when tested for growth in different pH and temperature. All 10 isolated bacteria showed positive reaction for pH 4, pH 6.5 and pH 7. These indicate that the LABs were able to grow in acidic or neutral pH because the original condition of *tempoyak* is indeed quite acidic. LABs that were successfully isolated from this homemade *tempoyak* were also found to be able to grow at 15ºC while there was no growth in temperature of 45ºC.

However, they demonstrated different reactions for growth in different concentration of sodium chloride (NaCl) as demonstrated in Table 1. LAB 3, 4, 5, 6 and 10 demonstrated negative reaction for all growth in 2%, 4% and 6.5% of sodium chloride. Meanwhile the other five LABs showed positive result either in 2% or 4% of sodium chloride. Growth of bacteria in different concentration of sodium chloride can be observed by development of turbidity after incubation for 1 week. Some bacteria are able to grow in 2% or 4% of sodium chloride but none of the bacteria can grow in 6.5% of sodium chloride.

Similarly, results shown for oxidative fermentation and biochemical sugar tests were different for the 10 LABs. This allows for determination of the LAB species. Different species may show different ability to utilize sugar carbohydrates as their source. About 19 sugar carbohydrates were prepared such as arabinose, D-mannitol, galactose, raffinose, glucose, lactose, maltose, sucrose, sorbitol, fructose, cellobiose, trehalose, salicin, esculin, rhamnose, mellobiose, melizitose, mannose, and xylose. Confirmation of the identified species was then carried out using API 50 CH in conjunction with API 50 CHL medium for the identification of *Lactobacillus* species and related genera. Based on the results obtained, three out of the 10 LABs have been identified as *L. plantarum* which were LAB 1, 3 and 9. LAB 2 was identified as *Lactobacillus brevis*, LAB 4 as *Lactobacillus fermentum*, LAB 5 as *Lactobacillus pentosus*, LAB 6 as *Lactococcus lactis* while LAB 7, LAB 8 and LAB 10 were identified as *Lactobacillus raffinolactis*, of *Lactobacillus paracasei* and *Leuconostoc mesenteroides*, respectively.

Table 2 demonstrated that effect of pH of *tempoyak* towards colony count during the 6 day fermentation period. The initial pH measurement of *tempoyak* after addition of 0.85% sterile saline was 6.65 and this coincide with the pH reading reported by Leisner et al. [11] which stated that the initial pH of *tempoyak* was 6.7. The pH reading dropped afterwards to 3.85 for day 1 and 3.87 and 3.93 for day 2 and day 3, respectively. Reduction in pH during day 1 was probably due to the fermentation process which involved utilization of carbohydrates to produce lactic acid and acetic acid by LAB. During this time, *tempoyak* became sour and acidic. However, the pH increased in day 4 to day 6 (pH 4.01 and pH 4.13). Values obtained were in agreement with the final pH reported by Merican [23]. According to Rathore, Salmeron and Pandiella [24], rapid drop in pH fermentation was due to the accumulation of lactic acid produced from metabolic pathway and as well as due to the energy requirement that LAB used for preservation of cell viability. However, the colony counts were inversely proportional to the value of pH which means that a decrease in pH value will increase the number of the colony count. During day 0 there is no growth of LAB colony on MRS agar but the number of LAB colony increases for day 2 and day 3 and decrease afterwards. However, after day 5 and day 6 the colony count is increase and this is because there may have more than one of LAB species which have different pattern of growth. Some species were able to grow at the first day of fermentation and another species showed delay growth at the later stage of fermentations. The highest colony counts were counted when the pH value decreased. Hence, the acidic taste of *tempoyak* might be due to the growth of LAB in *tempoyak*. In this study, the LAB colony count were found to vary in the range between 5.3×10^{10} to 1.24×10^{12} cfu/ml. The LAB count declined at the later stage of fermentation due to rapid increase in total acidity and decrease in pH value as suggested by Doyle, Beuchat and Montville [25].

5. CONCLUSION

Overall this study describes the process of isolation of LAB in homemade *tempoyak* until enumeration and identification of bacteria isolated. Ten of the isolated bacteria have been recognized and identified as LAB using biochemical characterization. Three of the LABs isolated were confirmed as *L. plantarum* which showed highest frequency of occurrence in *tempoyak*. Other species successfully recognized were *L. plantarum, L. brevis, L. pentosus, L. paracasei, L. raffinolactis, L. fermentum, L. lactis* and *Leuconostoc mesenteroides*. During fermentation of the *tempoyak*, lactic acid produced by LAB resulted in lowering of the pH which made the conditions inhospitable for many pathogenic microbes and inhibit their growth.

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