Antimicrobial activity of lactic acid bacteria isolated from fermented durian flesh (tempoyak) against pathogenic and spoilage bacteria during storage

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Abstract. Traditional fermented food ‘tempoyak’ (fermented durian flesh) origin Jambi, Indonesia was used as sources for the isolation of lactic acid bacteria (LAB) and also to study their antimicrobial activity and physicochemical properties of tempoyak during four weeks of storage against undesirable bacteria, which were performed using an agar well diffusion, assay method. An isolates were obtained and by sequential screening for catalase activity and gram-staining. The aims of this research were to obtain the total LAB isolates that have highest inhibitory activity against bacterial pathogens and spoilage (Staphylococcus aureus and Escherichia coli ATCC 25923) during four weeks storage and also the physicochemical properties of tempoyak was also investigated. The Completely Randomized Design (CRD) method was used, four replicates with storage times were 1, 2, 3, 4 weeks respectively. The results show that the antimicrobial activity of isolates inhibited the growth of Staphylococcus aureus and Escherichia coli ATCC 25923. There was a significant effect on total titratable acidity, moisture content, pH and total soluble solids content of tempoyak during four weeks storage. The result of this research illustrated that these LAB can be used widely in the food industry as bio preservatives due to their large inhibition spectrum.

1. Introduction

Tempoyak is traditionally fermented food from durians flash, which is a popular acid-fermented condiment, used with certain fish and vegetable dishes in ASEAN countries, including Jambi Indonesia. Durian flash is traditionally fermented through spontaneous and uncontrolled processes these will lead to variable quality, as the indigenous micro-flora is not consistent [1].

Durian pulp from different durians is mixed with or without addition of salt and allowed to ferment in tightly container at room temperature for a minimum of seven days for the development of acidity and flavour [2]. Lactic acid bacteria (LAB) play a very important role in food fermentations where they not only contribute to the development of the desired sensory properties in the final product but also to their microbiological safety of food product [3]. LAB are predominant microorganisms present in tempoyak ranging from 8.4 to 9.2 log CFU/g of tempoyak [4]. They had reported that lactobacillus plantarum were the predominant members of the LAB flora in tempoyak.

The antimicrobial activity of LAB is mostly related to the production of organic compound such as lactic- and acetic-acids, as well as propionic-, sorbic-, benzoic-acids, hydrogen peroxide, diacetyl, ethanol, phenolic- and proteinaceous-compounds however some strains are able to synthesize antimicrobial substances like bacteriocins [5].

Previous studies on tempoyak mainly only focused on the microbial aspect of tempoyak [6] [7] [8]. The effect of their antimicrobial activity and physicochemical properties of tempoyak during four weeks storages, however, have not been reported. The antimicrobial activities of tempoyak during
storage were determined against bacterial pathogens and spoilage agents, Staphylococcus aureus and Escherichia coli ATCC 25923.

The aims of this research were to obtain the LAB isolates that have highest inhibitory activity against pathogenic and spoilage bacteria (Staphylococcus aureus and Escherichia coli ATCC 25923) and a further objective was to evaluate the physicochemical properties of tempoyak and the viability of BAL during four weeks of storage.

2. Material and Methods

2.1. Materials

All chemicals used for analytical procedures were analytical grade or the highest purity available. Details of chemicals and suppliers were MRSA (Merck), MRS Broth (Merck), APDA (Merck), RCA (Merck), HCL, NaCL, NaOH, CaCO3, hydrogen peroxide and Gram test kits.

Staphylococcus aureus and Escherichia coli ATCC 25923 were obtained from the culture collection of Microbiology Laboratory of South Sumatera University.

2.2. Preparation of making tempoyak

Durian fruit was obtained from local market at Jambi, Indonesia. Fermented durian was prepared according to the local people method of fermentation. Durian pulp (200 g) was mixed with 2% of salt and was placed in sealed plastic containers. The durian was allowed to spontaneously ferment during four weeks storage at room temperature (24-31˚C). Microbiological and physicochemical analysis of the final products was performed after 1, 2, 3 and 4 weeks of storage in room temperature. Four replications were maintained in this experiment.

2.3. LAB Isolation and identifications

The LAB isolation method was according to Hayakawa (1992) [9] with some modifications. Tempoyak was made 2%, was taken each of samples and blended with 100 ml of 0.85% NaCl solutions aseptically; 10 ml of this blended food was added to 100 ml MRS broth (de Man et al, 1960) in a 250 ml erlemeyer flask. The flask were placed in an incubator for 48 hours at 37˚C. Then aliquots of the culture from each flasks were diluted serially to 106 times and 0.1 ml was spread evenly on MRS agar plates. The plates were incubated for 48 hours at room temperature. Bacterial colonies that developed on the plates were individually picked and streaked on fresh MRS agar plates by dilution streaking to obtain single colonies. This procedure was repeated in order to purify the isolates. Each of the isolates was tested for catalase by placing a drop of 3% hydrogen peroxide solution on the cells. Immediate formation of bubbles indicated the presence of catalase in the cells. Only those isolates, which were catalase negative, were Gram-stained [10]. The stock cultures were maintained on De Man Rogosa Sharpe (MRS) agar and stored at 4˚C

2.4. Determination of antimicrobial activity of LAB isolated from Tempoyak

The ability of LAB strains to produce antimicrobial metabolites was tested by an agar well diffusion assay by Schillinger and Lücke (1989) [11]. The LAB were propagated and growing in a shaken incubator at 37 C on MRS Broth media for 48 hours at their optimal temperatures. The LAB cells were harvested by centrifugation (10000 rpm) after growing, to prepare expected inoculums containing 108cell ml of LAB.

2.5. Physicochemical analysis

2.5.1. Total Titratable Acidity

Total titratable acidity (TTA) was determined following dilution of 10 g of the sample with 250 mL of distilled water which was then titrated with 0.1 mol/L NaOH (Merck), using a phenolphthalein indicator to faint pink end point persisting 30 s [12]. The results were reported as the percentage of lactic acid. 

\[([\text{ml NaOH} \times 0.1 \text{ N/ volume of sample titrated}] \times 0.09 \times 100)\]
2.5.2. Moisture content
Moisture content (MC) was determined gravimetrically by drying in the oven at 105°C until a constant weight was achieved [13].

2.5.3. pH
The pH was measured using a pH meter. About 1 g of each sample was added to 20 mL of distilled water, homogenized for 30 s, and the pH was then measured. Calibration was performed using standard buffers provided by the manufacturer at pH 4, and 7 at room temperature [13].

2.5.4. Total Soluble Solids Content (TSSC)
Total soluble solids were determined using a refractometer (Atago RX-1000, Atago Company Ltd., Tokyo, Japan) and expressed as °Brix [14].

2.6. Determination of LAB cellular viability
The viability of BAL was monitored by counting of viable cell for four weeks of storage. Samples of 1 g of tempoyak each week were added to 9 mL of 0.1% (w/v), sterile peptone-water. After this time, decimal dilutions were performed in 0.1% peptone-water followed by inoculation in MRS-agar and incubation at 37°C for 48 h. The number of viable cells was determined by plate counting, and results were recorded as colony forming units (CFU) per gram [15].

2.7. Statistical Analysis
The statistical analyses were performed using one-way analysis of variance (ANOVA). Excel software was used to perform statistical analysis, when significant differences were detected; the differences among the mean values were determined by performing the Duncan’s multiple comparison test at a confidence level of p < 0.05 [16]. Mean values and standard deviation of the mean are reported.

3. Result and Discussion

3.1. LAB Isolation and identification
Based on the results showed that all of LAB isolated from Tempoyak Jambi origin shows the characteristics of spherical-shaped colonies, milky white color of the colonies, positive Gram (+), negative catalase reaction, short rod-shaped cell with dark purple color and were tabulated in Table 1.

| Storage (Week) | Form | Gram | Catalase test |
|---------------|------|------|---------------|
| 1 | Rods, cocci | (+) | (-) |
| 2 | Rods, cocci | (+) | (-) |
| 3 | Rods, cocci | (+) | (-) |
| 4 | Rods, cocci | (+) | (-) |

The isolation, identification and screening of microorganisms from natural sources have always proved to be a successful way for obtaining industrially important strains or strains with valuable industrial and medical applications [17].

3.2. Determination of antimicrobial activity of LAB isolated from Tempoyak
The inhibitory activity against Staphylococcus aureus and Escherichia coli ATCC 25923 during four weeks storage of tempoyak are presented in Table 2.

**Table 2.** Inhibitory activity against Staphylococcus aureus and Escherichia coli ATCC 25923 during four weeks storage of tempoyak.

| Storage (Weeks) | Zone of inhibitions/mm |
|-----------------|------------------------|
|                 | **E. Coli** | **S. Aureus** |
| 1               | 16.25±0.95a  | 18.75±1.70a |
| 2               | 17.25±1.89a  | 19.00±1.41a |
| 3               | 17.50±2.51a  | 19.00±1.82a |
| 4               | 16.00±0.81a  | 18.25±1.25a |

The LAB strains supernatants effectively inhibited the growth of Staphylococcus aureus and Escherichia coli ATCC 25923 strains in varies degree (the diameters of the inhibition zones varied between 16.00 to 17.50 mm and 18.28 to 19.00 mm respectively). Highest antimicrobial activity show against S. aureus with inhibition zone diameter was 19.00 mm until week 3. Zone of inhibition of LAB against A. Staphylococcus aureus. B. Escherichia coli ATCC 25923 are shown in Figure 1.

![Figure 1. Zone of inhibition of LAB against. (a). *Staphylococcus aureus*. (b). *Escherichia coli ATCC 25923.*](image)

There was study show that Lactobacillus alimentarius, Lactococcus lactis subsp. lactis, Leuconostoc citreum and Lactobacillus plantarum producing acids were able to inhibit some microorganism [18]. The search and application of a new LAB with a wider spectrum of antimicrobial activities which can be applies in human health, and food industry is being studied by several groups [19] [20].

### 3.3. Physicochemical properties of tempoyak

The physicochemical characterizations of the tempoyak properties during four weeks of storage are presented in Table 3. Based on Table 3 shows tempoyak have characteristics different range of TTA, MC, pH, and TSSC during four weeks of storage.

There was a significant difference in the pH during storage. From Table 3 it can be seen that there was a reduction in pH from week 1 to week 3 and increased in week 4 of storage. The lowest pH was recorded at week 3. Reduction in pH during fermentation and storage of tempoyak is of paramount importance for maintaining the quality of tempoyak. This agrees with the results was reported by Viander et al. [17] that pH reduction is of great importance for the quality of the end product. There is a changes in the pH and increase in lactic acid development during fermentation and storage of tempoyak may be due to the metabolic activity of the LAB.
Table 3. Physicochemical properties of tempoyak from four weeks storages.

| Storage/ Weeks | TTA* (%) | MC* (%) | pH* | TSSC* (Brix) |
|----------------|----------|---------|-----|--------------|
| 1              | 17.48±0.87b | 45.85±1.40a | 4.24 ±0.06c | 3.05±0.26c |
| 2              | 19.03±1.42b | 54.08±1.23bc | 4.04±0.09b | 2.58±0.05b |
| 3              | 21.61±1.52c | 55.73±0.91c | 3.90±0.07a | 1.98±0.09a |
| 4              | 14.12±2.08a | 53.72±0.81b | 4.12±0.09bc | 1.83±0.09a |

Means with different superscript letters in the same column indicate significant differences (p 0.05) between the carriers.

From Table 3 also it shows that there was a significant difference in the TTA production during the storage time. There was an increased in TTA from week 1 to week 3 and decreased rapidly after week 3. The highest TTA was recorded at week 3. The increase in the acid equivalent increase in lactic acid and storage may be due to the metabolic activity of the LAB. The increase in TTA will inhibit the activity of the spoilage microorganisms. The similar result was also reported by Gaanappriya et al. [21]. Karovicova and Kohajdona [22] claimed that lactic acid fermentation can be suppressed by bacteria activity in a slowly acidified medium.

The TSS assessment of the tempoyak samples during four weeks of storage is shown in Table 3. There were significant differences (p 0.05) in the decreased of TSS during storage. The lowest TSS was recorded in the fourth week. The reduction in the TSS during fermentation and storage may be due to the utilization of sugars and other metabolic activity by LAB in the tempoyak. The similar result was also report by Kumar et al. [23], who observed a similar reduction in juice with role of LAB.

3.4. LAB cellular viability
There is no significant difference in the LAB cellular viability during storage however still have a high viable count (>107 cfu/mL) after 4 weeks of storage. The LAB viability during four weeks storage of tempoyak are presented in Table 4.

Table 4. LAB viability during four weeks of storage.

| Storage (Week) | LAB* (Log CFU/mL) |
|----------------|-------------------|
| 1              | 7.59a             |
| 2              | 7.54a             |
| 3              | 7.53a             |
| 4              | 7.28a             |

A high viable count after 4 weeks of storage is important for maximum health benefits. Furthermore, the LAB culture should be able to multiply to reach high cell counts in the fermented product and possess a high acid tolerance to ensure high viable cell numbers during storage. The viability of probiotic organisms is could also dependent on many factors, such as the level of oxygen in products, oxygen permeation of the package, fermentation time, and storage temperature [24].

4. Conclusions
In conclusion, there was a significant effect of the LAB isolated from tempoyak into physiochemical their properties during four weeks of storage. The LAB isolated from tempoyak can be used widely in the food industry as bio preservatives due to their large inhibition spectrum and strong inhibitory activity against Staphylococcus aureus and Escherichia coli ATCC 25923.
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