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Isolation and Identification of Lactic Acid Bacteria Using PCR Gene from Tempe Wrapped with Banana Leaves and Plastic

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***Abstract*— Tempe is a typical Indonesian food that comes from fermenting soybeans with the fungus Rhizopus sp. Tempe is known to have good nutritional value because of the content contained in soybeans themselves and other microorganisms that appear as a result of the tempe fermentation process. The fermentation process increases the activity of bacteria in tempe which are beneficial for digestion, one of which is lactic acid bacteria. This research aims to see the differences morphological forms and genomic of lactic acid bacteria produced by tempe wrapped in banana leaves and tempe wrapped in plastic. The differences in fermentation that occurred in tempe wrapped in banana leaves against those wrapped in plastic resulted in variances in lactic acid bacteria type. Based on the isolation of bacteria on Mann de Rogosa Sharpe Agar medium, 15 isolates of lactic acid bacteria were produced, with general morphological forms of bacilli and coccus. The PCR was then used to identify genomic sequences. The phylogenetic tree generated with the 16S rRNA gene sequence can demonstrate the relationship between LAB diversity at the species level, but it cannot identify LAB from the strain level.**

***Keywords*— Fermentation; Lactic Acid Bacteria; Probiotics; Sequencing; Tempeh.**

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I. INTRODUCTION

Tempeh is a traditional food fermented from the activity of the fungus Rhizopus sp. Tempeh is one such product and will be the focal point of attention in this review. Tempeh also referred to as tempeh, is the collective name for a sliced mass of cooked mushroom fermented beans, cereals, or other food processing byproducts bound together by the mycelium of live fungi (mostly Rhizopus spp.) [10].

Indonesia is the largest country in tempeh production in the world. Currently, in Indonesia, there are around 81 thousand tempe-making businesses that produce 2.4 million tons of tempeh per year. The tempeh industry produces around Rp. 37 trillion added value. From data held by the Indonesian Tofu and Tempe Cooperative Primer (Primkopti), of the 2.2 million tonnes per year of domestic soybean demand, only 600 thousand tonnes can be met by local soybean farmers [16].

Tempeh is a soy-based product, which has benefits both in terms of nutrition and health. Tempeh is a source of nutrition that contains 25% protein, 5% fat, 4% carbohydrates and is rich in minerals and vitamin B12. Several studies show that tempeh nutrients are easier to digest, absorb and utilize by the body compared to soybean nutrients consumed directly [5]

The open type of fermentation in tempeh production allows microbial communities that are obligate aerobes or facultative anaerobes from the environment with Rhizopus spp. as yeast, hydrolyzes complex soybean compounds into simple compounds that are more easily digested and increase their nutritional value [8];[11];[4]. Microbial activity during

the fermentation process plays a role in the transformation of food ingredients so that it can increase the nutritional content and taste [3] Due to its substance content, tempeh has the ability to increase the composition of beneficial bacteria in the human intestine [16]. The presence of lactic acid bacteria in the microbial community makes tempeh a source of probiotics that can increase the immune response [3]

Probiotics are microorganisms with potential beneficial effects on the health of the host organism. LAB is a type of beneficial bacteria, because it plays a role in the world of food and health. LAB is included in the group of bacteria that meets Generally Recognized As Safe (GRAS) status for humans [10].

One way that can be used to identify microorganisms is to use the 16S rRNA gene. One is through analysis of the 16S rRNA gene. The reason for using the 16S rRNA gene sequence is that it is universal, highly conserved, and exists in all bacteria. This technique is easier to perform, faster, and allows for the analysis of phylogenetic relationships with quite distant taxa [15]. The 16S rRNA gene has been widely used to detect and study the genetic diversity of bacterial groups in a habitat. Therefore, it can be used to identify lactic acid bacteria.

The aim of this research is to identify and compare LAB (morphological form and genomic) produced from tempeh wrapped in banana leaves and plastic packaging.

II. MATERIAL AND METHODS

A. *Materials*

The materials used in this research were tempeh samples obtained from traditional markets in Padang, Indonesia with various packaging treatments, alcohol, 0.9% NaCl, banana leaf (*musa paradisiaca*) , plastic wrapping (polyethylene plastic (PET), and hypochlorite solution, Medium deMan Rogosa Sharpe (MRS) agar. For bacterial staining, crystal violet, Lugol, safranin and 70% alcohol were used, primer forward 27F (5'-AGAGTTTGGATCCTGGCTCAG-3'), primer reserve 1492R (5'-GGTTACCTTGTTACGACTT-3') Bacto agar, DNA ladder 1 kb Generuler, agarose 1%, sybr safe DNA gel strain.

B. *Procedure*

1) Sample Preparation

Tempe which obtained from traditional markets, then stored for three days in the room temperature. Followed by grinded, and serial dilutions. Serial dilutions were carried out using distilled water starting from a dilution of 10^{-2} g/mL to a dilution of 10^{-6} g/mL.

2) Lactic acid bacteria isolation

Lactic acid bacteria were isolated from each samples. The medium used for LAB isolation is de-Man Rogosa Sharpe (MRS) [14]. LAB isolation was carried out by means of graded dilutions up to $10^{-6.}$ A total of 100 µL of the diluted suspension was taken using a micropipette and inoculated onto the surface of the MRS agar medium. The inoculum was spread using a spread plate method using a Drigalski and incubated in an incubator at 37°C for 2x24 hours.

3) Identification of LAB

LAB identification is carried out macroscopically and microscopically. Macroscopic observation in the form of visualization of the morphology of a single bacterial colony. The aspects observed are the shape, color, edges and elevation of the bacterial colony. Microscopic observations were carried out using the gram staining technique [1]. The stained bacteria are observed under a microscope to see the cell shape and gram type [13].

The 16S rRNA gene of lactic acid bacteria isolates was amplified using universal primers 27F (forward) and 1492R (reserve)(Tajabadi et al, 2011). The PCR reaction was carried out in 50 µl reactions, consisting of 7 µl ddH2O, 1 µl Kod Fx Neo (Toyobo), 1 µl each of forward and reverse primers of a concentration of 20mM, 5 µl DNA template, 10 µl 2mM dNTP mix and 25 µl 2x PCR buffer. The temperature cycle used was initial denaturation at 94ºC for 2 minutes, followed by 30 cycles consisting of denaturation at 98ºC for 10 seconds, primer attachment (annealing) at 53ºC for 30 seconds, and elongation at 68ºC for 1 minute 30 seconds. The reaction was closed with a final elongation of 68ºC for 7 minutes.

Type identification was carried out by aligning the 16S rRNA gene sequence resulting from sequencing with GenBank data using the program (Basic Local Alignment Search Tool-Nucleotide (BLAST-N) from the NCBI website. BLAST-N was carried out to determine the similarity of the species of the bacterial isolate tested to the bacterial sequence found in GenBank data.

III. RESULT AND DISCUSSION

A. Tempeh Fermentation

The tempeh samples that were obtained were further fermented for 3 days (**Figure 1**). During this period, changes occur in the wrought iron, where the color becomes browner and emits an odor. Tempeh wrapped in banana shown more spoilage than tempeh wrapped in plastic, this is because tempeh wrapped in banana leaves is not completely wrapped, so there are still some parts of the tempeh that are in contact with the outside environment. This is in accordance with research conducted [3], where tempeh wrapped in plastic has a smaller chance of spoilage than tempeh wrapped in banana leaves.

Fig. 1. Tempeh image with wrapped in banana leaves (a) and polyethylene plastic (PET)

B. Total Number of LAB from Tempeh

The ground tempeh was then diluted, after which bacterial isolation was carried out by taking 100-100 µL of the suspension resulting from the tempeh dilution, two repetitions were carried out for each type of tempeh. Bacteria that grew on de-Man Rogosa Sharpe agar medium, as shown in **Figure 2** below:

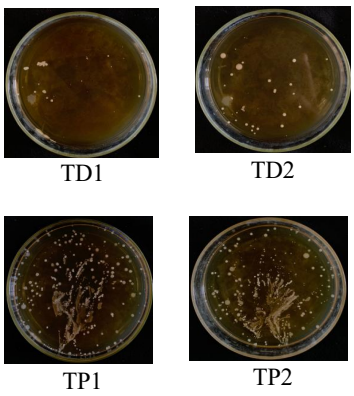

Fig. 2 : Lactic acid bacteria isolation from leaves wrapped tempeh 1(TD1), leaves wrapped tempeh 2(TD2), plastics wrapped tempeh 1(TP1), plastics wrapped tempeh 2(TP2).

Then the number of bacteria was counted using the total plate count method. Tempeh leaf samples had fewer bacteria than tempeh wrapped in plastic. The results of calculating the number of bacteria can be seen in **Tabel 1**.

TABLE I
TOTAL NUMBER OF BACTERIA IN TEMPEH

Code	Total LAB Count (cells/mL)
TD 1	$8{,}3X10^7$
TD 2	$6{,}3X10^7$
TP 1	$70{,}3X10^7$
TP 2	$43{,}5x10^7$

This significant difference in the total number of bacteria is one of the factors caused by the protein content of tempeh itself. Protein is an important substance for the body because this substance, besides functioning as fuel in the body, also functions as a building agent and regulating agent. Protein can be used as fuel if the body's energy needs are not met by carbohydrates and fat [18]. The presence of protein in tempeh is also influenced by the packaging in which the tempeh is wrapped. [4], stated that the type of packaging also influences the protein content in tempeh. Tempeh leaf packaging has a high protein content because it has air circulation that is suitable for the growth of the mold Rhizopus sp. so that the fermentation process can run well. In this study, tempeh wrapped in leaves had too open air circulation so that the tempeh used actually rotted. The more optimal the growth of mold is, the more optimal the work of the protease enzyme will be to break down protein into free amino acids, causing differences in protein levels.

C. Isolation and Identification of LAB from Tempeh

Based on the isolation and identification carried out, 15 LAB isolates were obtained from 2 types of tempeh. 10 LAB isolates came from tempeh wrapped in leaves, while 5 more isolates came from tempeh wrapped in plastic. And from this isolation, 12 isolates of lactic acid bacteria with a round shape (Coccus) were obtained, while the other 3 isolates had a stem cell shape (Bacil). Lactic acid bacteria are classified based on cellular morphology, glucose fermentation method, growth temperature range, and sugar utilization patterns [9]. Lactic acid bacteria (LAB) are divided into 12 genera, namely: Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus and Weissella [8].

This difference in tempeh packaging is likely to be a factor in the differences in BAL produced. Because the difference in wrapping will affect the fermentation carried out by the tempeh. The source of nutrition is based on research, testing the compound components produced by tempeh wrapped in leaves are not significantly different, but there are several differences in concentration between the two [14]. Lactic acid bacteria (LAB) make an important contribution to the fermentation process and ensure the safety of the tempeh produced [6].

Apart from being influenced by the tempeh packaging, raw material factors, yeast selection, tempeh making process and packaging method will influence the final results and tempeh fermentation. According to the standard packaging process is packed in well-closed packaging and the process of making holes in the tempe packaging at a distance of 2x2 cm to help balance oxygen exchange during the fermentation process [18]

TABLE 2
MICROSCOPIC LAB IDENTIFICATION

Code	Macroscopic Identification	Microscopic Identification
TD1.10^{-6} 1	Shape : *Circular* Edge : *Entire* Elevation : *Convex* Color : White	Gram : Positif (+) Shape : Coccus
TD1.10^{-6} 2	Shape : *Circular* Edge : *Entire* Elevation : *Convex* Color : Crème	Gram : Positif (+) Shape : Coccus
TD1.10^{-6} 3	Shape : *Irregular* Edge : *Undulate* Elevation : *Flat* Color : Grayish	Gram : Positif (+) Shape : Bacill
TD1.10^{-6} 4	Shape : *Irregular* Edge : *Lobate* Elevation : *Raised* Color : White	Gram : Positif (+) Shape : Coccus
TD1.10^{-6} 5	Shape : *Circular* Edge : *Entire* Elevation : *Convex* Color : White	Gram : Positif (+) Shape : Coccus
TD2.10^{-6} 1	Shape : *Circular* Edge : *Entire* Elevation : *Flat* Color : White	Gram : Positif (+) Shape : Coccus
TD2.10^{-6} 2	Shape : *Circular* Edge: *Filamentous* Elevation : *Flat* Color : White	Gram : Positif (+) Shape : Coccus
TD2.10^{-6} 3	Shape : *Irregular* Edge : *Entire* Elevation : *Pulvinate* Color : White	Gram : Positif (+) Shape : Coccus
TP.110-6 1	Shape : Circular Edge : Entire Elevation : Raised Color :White	Gram : Positif (+) Shape : Bacill
TP.110-6 2	Shape : Circular Edge : Entire Elevation : Flat Color: Grayish	Gram : Positif (+) Shape : Coccus
TP.210-6 1	Shape : Circular Edge : Entire Elevation : Flat Color :White	Gram : Positif (+) Shape : Coccus
TP.210-6 2	Shape : Circular Edge : Entire Elevation : Raised Color :White	Gram : Positif (+) Shape : Coccus
TP.210-6 3	Shape : Circular Edge : Entire Elevation: Convex Color:Grayish	Gram : Positif (+) Shape : Bacill
TD 2.10-6 4	Shape : Circular Edge : Entire Elevation: Convex Color :White	Gram : Positif (+) Shape : Coccus
TD 2.10-6 5	Shape : Circular Edge : Entire Elevation: Convex Color : White	Gram : Positif (+) Shape : Coccus

Isolation and identification of tempeh leaves and plastic tempeh resulted in 15 different LAB isolates which were successfully isolated from tempeh wrapped in leaves and tempeh wrapped in plastic, coded as TD1, TD2, TP1, and TP2. Tempeh samples packaged in leaves had fewer bacteria than tempeh samples packaged in plastic. Soybean tempeh contains flavonoid compounds. The greater antioxidant activity of soybean tempeh compared to soybeans is thought to be due to the increase in total flavonoid levels after soybeans are processed into tempeh [2] Various types of nuts have been studied by [20]. regarding the potential of antioxidant compounds.

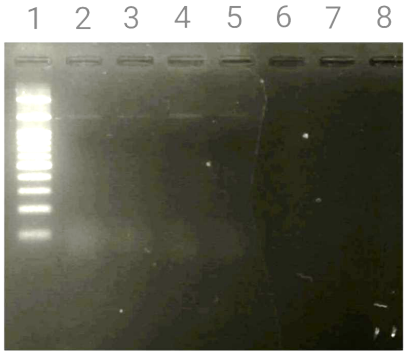

Fig. 3. Electroforensis image

Conventional polymerase chain reaction (PCR) applications Information:
1 = Marker DNA ladder 100 kb 4 = sampel (TD 2 10^{-6} 5)
2 = sampel (TD 2 10^{-6} 2)
3 = sampel (TP 2 10^{-6} 3)

Flavonoids are a source of natural antioxidants. flavonoids cause damage to the permeability of bacterial cell walls, microsomes, and lysosomes through the interaction between flavonoids and bacterial DNA. The lipophilic nature of flavonoids causes damage to the bacterial cell membranes. [15]. This research was carried out with a PCR process that began by entering 15 µL of master mix into the PCR tube, 1.2 µL of forward primer and reverse primer each, 1 µL of DNA template, and 11.6 µL of Nfw. In the PCR method, there are four stages, namely predenaturation, denaturation, primer attachment (annealing) and elongation of the amplified DNA strand (extension). Predenaturation is carried out at a temperature of 95℃ for 2 minutes. This aims to further stabilize the enzymes in the mastermix so that they can work optimally before the PCR process. Denaturation is carried out at 95^{0}C for 5 seconds. This process opens the

DNA double helix strand by breaking the hydrogen bonds that connect the two. Combination of annealing and extension at 53℃ for 10 seconds. The best annealing temperature is usually 2-5 °C below Tm. Tm is the temperature at which half of the DNA molecules experience denaturation. Too high a temperature causes the attachment of specific primers but the amplicon concentration obtained is very small so the annealing temperature is very critical in the target DNA amplification process. Meanwhile, a temperature that is too low causes the target DNA band whose results are expected to be non-specific. The factors that influence less specific bands are, DNA tamplates Poor integrity, Low purity, Insufficient quantity, Long targets. Primers, Problematic design, Old primers, insufficient quantity. Other reaction components, Inappropriate DNA polymerase, Insufficient quantity of DNA polymerase. Thermal cycling conditions, Suboptimal denaturation, Suboptimal annealing, Suboptimal extension, Suboptimal number of PCR cycles.

IV. CONCLUSION

The differences in fermentation that occurred in tempe wrapped in banana leaves and those wrapped in plastic resulted in differences in lactic acid bacteria type. Based on the isolation of bacteria on Mann de Rogosa Sharpe Agar medium, 15 isolates of lactic acid bacteria were produced, with general morphological forms of bacilli and coccus. The phylogenetic tree built based on the 16S rRNA gene sequence shown the relationship between LAB diversity at the species level, but cannot differentiate LAB from the strain level.

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CONFLICT OF INTEREST

Authors declare no conflict of interest to disclose.

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