FERMENTATION OF TOFU USING CONSORTIUM OF LACTIC ACID BACTERIA ISOLATED FROM TOFU WHEY AS BIOCOAGULANT AND BIOPERSERVATION

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Abstract

Tofu is a traditional food high in protein and is prone to spoilage. Fermentation process using a consortium of lactic acid bacteria (LAB) that can produce bacteriocin was carried out to get tofu with long shelf life and better taste. The bacteria used in this study were *Lactobacillus fermentum, Leuconostoc mesenteroides,* and *Lactobacillus plantarum.* The *L. fermentum* was isolated from tofu whey. Antibacterial activity was measured by conducting bacteriocins assay. The antibacterial activity for each bacterium was 0 AU/mL, 2506.429 AU/mL, 1502.679 AU/mL (*L. fermentum*); 0 AU/mL, 2506.43 AU/mL; 2939.44 AU/mL (*L. mesenteroides*); and 760.39 AU/mL, 3341.25 AU/mL, 3889.29 AU/mL (*L. plantarum*) after incubation for 0 hour; 4 hours; and 8 hours respectively. Ratio of bacteria inoculum (*L. fermentum: L. mesenteroides: L. plantarum*) were 2:1:1, 1:1:2, and 1:1:1 by using 10% of inoculum. The optimal inoculum age of these bacteria was 6 hours with μmax for *L. fermentum, L. mesenteroides, and L. plantarum* was 0.04 h^{-1}, 0.02 h^{-1}, dan 0.02 h^{-1} respectively. The fermentation was processed for 8 hours at 37^0C of temperature without agitation. From this optimation, the highest bacteriocin activity in tofu curd was 1767.857 AU/mL, which was in a ratio of 1:1:2 (*L. fermentum: L. mesenteroides: L. plantarum)* at the 4th hour. Organoleptic tests showed that tofu with LAB as coagulant had better taste than conventional tofu.

Keywords: LAB, *Lactobacillus fermentum*, bacteriocin, fermentation, tofu

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Introduction

Tofu is a traditional food which is very popular in Indonesia. Food with high protein content is prone to spoilage, including tofu. Bacteria that often contaminate tofu are from the genera *Bacillus*, lactic acid bacteria such as *Streptococcus* and *Leuconostoc*, and coliform which is resistant to refrigeration temperatures (Hermayani, 2010). This condition makes tofu has short shelf life. In order to prolong the shelf life, additional preservatives are often added to tofu. Research and development of natural preservatives derived from plants, animals, and microorganisms are increasingly being investigated. One of the natural ingredients used and tested is bacteriocin derived from lactic acid bacteria (LAB) (Ray, 1992).

In the process of making tofu, coagulation or clotting is the most important stage of the process. Coagulation is a complex process correlates to many chemical variables, such as total solids, pH, volume of soybeans, type, number and concentration of coagulant, method of adding and mixing as well as temperature and time of coagulation. In Indonesia, a commonly used method for clumping soy milk in making tofu is by adding tofu stone ($CaSO_4$). Another way is by fermentation method where whey is added and left for one day so that lactic acid bacteria can grow and produce lactic acid. The lactic acid then acts as the coagulant (Susanti, 1999). Compared to tofu stone, tofu whey has better taste and aroma and lower in acidity level. Whey of tofu contains several types of bacteria, including lactic acid bacteria. The lactic acid produced by lactic acid bacteria

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during the fermentation process causes the pH of soy milk to decrease and reaches the isoelectric point that coagulates the tofu protein.

Lactic acid bacteria (LAB) are bacteria found in food ingredients such as milk, meat, and other food ingredients that are easily damaged, and processed food ingredients (Rodriguez *et al.*, 2002). Lactic acid bacteria can maintain food quality from pests and spoilage bacteria by producing organic acids, hydrogen peroxide, diacetyl, fatty acids, and bacteriocins (Deegan *et al.*, 2005). Lactic acid bacteria have been used in food preservation and can modify food flavor, such as food aroma, taste, and texture. Various strains of lactic acid bacteria can be found in dairy products such as yogurt and cheese, fermented meat (salami), fermented vegetables (sauerkraut), and various other types of food (Florou-Paneri *et al.*, 2013). Lactic acid bacteria will produce organic acids at the beginning of fermentation, which will reduce the pH below 5.1 so that the characteristics of the food change and results in enrichment of taste, aroma, and texture of the final product. Lactic acid bacteria strains are also known to withstand acidic condition and high salt concentration (Zhang *et al.*, 2010).

Bacteriocins are proteins or peptides produced by various types of bacteria and these proteins/peptides are known to have anti-microorganism activity. Bacteria that are known to produce bacteriocins are a class of lactic acid bacteria. Most of the lactic acid bacteria produce bacteriocins with a broad antimicrobial spectrum, meaning that they can inhibit the growth of gram-negative bacteria as well as gram-positive bacteria. Therefore, bacteriocin can be applied in food industry as a natural preservative that can be used to replace artificial preservatives that are harmful to humans. With bacteriocin as a preservative, it is hoped that the use of harmful artificial chemical compounds and the heating process for preservation can be reduced. Reducing these two things allows food to be preserved naturally and have richer nutritional content. Public demand for safe, fresh, ready-to-eat, and not too much processed food can also be met (Galvez *et al.*, 2007). LAB starter culture's role in food safety is to suppress pathogens and microorganisms that can cause food spoilage, because lactic acid bacteria produce acids and bacteriocins (anti-microbial peptides). Through the production of both bacterial acids and bacteriocins, the growth of unwanted microorganisms can be suppressed without the addition of antibiotics (Mayo, 2007).

We conducted a study to obtain tofu with longer shelf life and better taste by using fermentation process with a consortium of lactic acid bacteria that can produce bacteriocins.

Materials and Methods

The starter was consisting of three types of bacteria inoculum: bacteria isolated from whey tofu, *Leuconostoc mesenteroides*, and *Lactobacillus plantarum* with the following composition variation: (2: 1: 1); (1: 2: 1); (1: 1: 2), and (1: 1: 1). These four variations were used to determine the effect of adding one inoculum more than the other, as well as increasing the number of inoculums in a balanced manner to the levels of lactic acid and bacteriocin in tofu, to get tofu which is more preferred and more preserved.

Media that were used in this study including whey tofu, MRS agar and MRS broth, and Nutrient Agar (NA). *Lactobacillus fermentum* culture was isolated from whey tofu obtained from a tofu factory in the Bengkok area, Dago Atas, Bandung, which was the waste from its tofu production. *Lactobacillus plantarum* and *Leuconostoc mesenteroides* were obtained from the culture stocks from Microbiology Laboratory, School of Life Science and Technology, Bandung Institute of Technology (SITH ITB).

Isolation of Bacteriocin-Producing Lactic Acid Bacteria from Tofu Whey The medium used in the isolation process was MRS Agar (MRSA). A sample of 1 mL of tofu whey was taken aseptically and added into 9 mL of physiological solution. The solution was then diluted to 10^{-3}. A total of 0.1 mL of homogeneous sample solution was inoculated using the scatter method (Cappucino and Sherman, 2001). The medium was incubated at 37^0C for 48 hours. Colonies formed on the agar surface were taken from one single colony, then were purified on a new MRSA medium by the friction method (4-way streak) to obtain a pure culture (Cappucino and Sherman, 2001). The cultures were grown for 24-48 hours and then stored at 4^0C.

Lactic Acid Bacteria Screening Five isolates were obtained from tofu whey by growing the bacteria on MRSA medium, which is a selective

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medium for lactic acid bacteria, using the spread method. Identification was carried out using Gram staining and a test to identify the presence of endosperm in the bacteria isolates was conducted. A smear of bacteria was deposited on a glass slide and thoroughly air-dried. It was stained for 1 min with crystal violet solution, 1 min with iodine solution, washed for 10 s in ethanol and finally, counterstained with safranin for 1 min. The glass slide was examined under oil immersion at 100× – 250× magnification with direct illumination using Dialux 20 microscope. All bacterial cells were stained with crystal violet and iodine, but only Gram-negative cells lost the color when alcohol was applied. Subsequently, these bacterial cells were stained with safranin dye. Gram-positive cells remain blue-purple (Madigan *et al.*, 2009). Gram-positive colonies were used for the initial bacteriocin activity test and were identified biochemically (Azadnia and Khan, 2009). Molecular identification analysis using 16S rRNA was carried out by Macrogen Singapore. Macrogen controlled and performed all processes from gDNA extraction of the bacteria to PCR amplification, purification, sequencing, and BI report. For bacteria, PCR of 16S rRNA genes was performed using 27F and 1492R primers, and sequencing was conducted using 785F and 907R primers, which were the inter-primers, to identify bacteria.

Inhibitory Activity Test Cultures were grown on MRS medium (pH 6.0). The culture was grown by taking 5% of the inoculum at 24 hours of age and incubated at 37^0C for 48 hours. After incubation, cells were separated from the medium by centrifugation (10.000xg for 15 minutes, 4^0C). Supernatant containing cells was adjusted to pH 6.0 using 1N NaOH and used as bacteriocin crud (Ogunbanwo, *et al.*, 2003).

Bacteria inhibitory activity was tested by using well diffusion method by inoculating the indicator bacteria and the tested bacteria in one culture. The indicator bacteria were *E. coli*, and the tested bacteria were activated LAB. NA medium contained *E. coli* indicator bacteria cells, which were inoculated by pouring method, was let stand for 30 minutes until it was hard enough. Then the wells were made by perforating the agar media with a hole punch with a diameter of 9 mm. A total of 100 µL of LAB supernatant was inserted into the well, then incubated at 37^0C for 48 hours and its activity was observed by measuring the area of the bacteriocin inhibition zone (clear zone) around the well. The bacteriocin inhibition zone is related to single hit inactivation, one bacteriocin molecule can kill an indicator bacterial cell (Ray, 1996). Bacteriocin activity is expressed as Arbitrary Units (AU) of the culture medium (Simonova and Laukova, 2007). One AU is the area of inhibition per unit volume of bacteriocin sample tested (mm^2 / mL) (Tagg and McGiven, 1971), written with the formula:

Bacteriocin Activity (mm^2/mL) = 1 AU/mL

$$= \frac{Lz - Ls}{V}$$

Lz = Area of clear zone (mm^2)
Ls = Well area (mm^2)
V = volume of sample (mL)

Inoculum Growth Analysis Preculture was carried out using MRS broth medium. One loop of culture was transferred to the MRS broth medium for activation and incubated for 24 hours at 37^0C. Ten percent of the activated solution was added to the second MRS broth and incubated for 24 hours at 37^0C. This activation is carried out for each bacteria that will be used in the study.

As much as 0.1 mL of the final activated solution was then taken and diluted using 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilution. The results of each dilution were inoculated by spread method into MRS agar medium in a petri dish to determine the number of activated bacteria. For optimum inoculum age determination, the bacterial cell density that would be inoculated into new medium was adjusted to 10^6 cells/mL.

The determination of the optimum inoculum age for each bacterium was done by making a growth curve. To make the growth curve, MRSB medium was used. A total of 3 mL of activated lactic acid bacteria culture with density of 10^6 cells/mL was added to 27 mL of MRSB medium. The culture in the MRSB medium was then incubated without shaking at 37^0C.

Sampling was done every 2 hours and at each sampling, the following procedures were carried out:

a. Total Plate Count (TPC) To count the number of bacteria, 1 mL of sample was put into a tube containing 9 mL of 0.85% NaCl. Then, a serial dilution was performed. Finally, 0.1 mL

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of the last three dilutions was inoculated on MRS agar medium and incubated for 48 hours at 37^0C. The number of grown colonies were observed under a microscope.

b. Determination of lactic acid levels The concentration of lactic acid was determined by titration method (Stryer *et al.*, 2002). A total of 5 mL of sample was added with 10 mL of distilled water and 5 mL of phenolphthalein and then was titrated by 0.1 M NaOH. The concentration of lactic acid can be obtained using the formula (Stryer *et al.*, 2002):

$$\% \text{ Lactic acid} = = \left[\frac{\text{mL NaOH x N NaOH x } (\frac{90}{1000})}{\text{mL sample}} \right] \text{ x } 100\%$$

c. pH testing is carried out using a pH-meter at room temperature.

From the data obtained, a bacterial growth curve was made by plotting the time (x-axis) to the log of the number of cells (y-axis). Based on the growth curve, the best inoculum age was then determined. At the optimum inoculum age, bacteria have the fastest and most active growth rates. The optimum inoculum age is chosen when the bacteria are at the ½ of logarithmic phase (Stryer *et al.*, 2002).

Optimization of Fermentation Using Variation in Inoculum Ratio The inoculums used were *L. fermentum*, *L. mesenteroides*, and *L. plantarum*. We tested 4 different inoculum ratio for *L. fermentum*: *L. mesenteroides*: *L. plantarum* which were (2: 1: 1); (1: 2: 1); (1: 1: 2); and (1: 1: 1). Determination of optimum inoculum age was done by making a growth curve. To make the growth curve, a soy milk medium was used. A total of 3 mL of activated lactic acid bacteria culture with a density of 10^6 cells/mL was added to 27 mL of whey tofu medium and 1% sucrose in 13 vial bottles. The culture in tofu whey medium was subsequently incubated without shaking at 37^0C. Then bacteriocin activity was measured.

Tofu Making The soybean used was local soybean, which was smaller than imported soybean usually found in supermarkets. The soybean was soaked overnight to make it easier to exfoliate the seed coat and to eliminate unpleasant odors. After the soybean expanded, the seed coat was peeled and washed. The soybean was added with water three times the weight of the soybean, then was mashed in a blender to obtain soy pulp. This soybean porridge is cooked to boil while stirring. The soybean slurry was then filtered with a cloth to get soy milk, which was then pasteurized. This soy milk was then coagulated to make tofu. The coagulant in this study was a consortium of LAB with variation in the ratio of inoculum. The tofu was stored at room temperature for seven days until it showed an indication of spoilage.

Tofu Organoleptic Test Organoleptic tests were carried out by asking 20 untrained panelists to try out the tofu product produced from the fermentation process. The test conducted was a preference test that involved a person's assessment of material characteristic. Organoleptic test relates to a person's liking or response to the sensory trait or quality being assessed (Soekarto, 1981). The parameters tested from the tofu were the taste, aroma, and texture of the tofu. There were six scales used in this organoleptic test, namely 6 = like very much, 5 = like, 4 = like moderately, 3 = neutral, 2 = dislike, 1 = dislike very much.

Results

Isolation of Bacteriocin Producing Bacteria from Tofu Whey Lactic acid bacteria can ferment sugar from carbohydrates to produce lactic acid in large quantities. The characteristics of lactic acid bacteria, in general, are Gram positive bacteria, respond negatively to catalase, and do not form spores and ferment glucose into lactic acid (Rahayu, 2003).

We obtained five isolates of lactic acid bacteria from whey tofu. The growing bacteria were observed, and the number of colonies were counted, then Gram staining and endosperm staining were carried out, with the observation results shown in Table 1.

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Table 1. Results of the isolation of lactic acid bacteria from tofu whey

Isolate	Colony characteristics	Number of Cells (cfu/mL)	Cell characteristics
A	Brownish cloudy, wide, there are peaks, the colony is very thick	$1,0x10^2$	Gram positive, oval cell shape, short chain, no endospores
B	Milky white, convex, shiny, 1-2 mm in diameter	$1,7x10^2$	Gram positive, oval cell shape, short chain, no endospores
C	Milky white, convex, shiny, very small diameter 1 mm	$3,0x10^3$	Gram positive, round cell shape, no endospores
D	Brown, thin flat, serrated	$3,0x10^3$	Gram positive, coccus, round cell shape, no endospores
E	Serrated, brownish, large size, transparent	$1,0x10^1$	Gram positive, round shape, quite long chain

Selection of bacteria with antimicrobial activity was carried out by using well diffusion method. This selection process used *Escherichia coli* as indicator bacteria which growth is inhibited by the LAB. Some studies suggest that bacteriocins are not only inhibit phylogenetically close species but are also able to inhibit Gram-negative bacteria. Ogunbanwo *et al.* (2003) stated that bacteriocins from *Lactobacillus brevis* could inhibit *E. coli, S. typhimurium,* and *L. monocytogenes*. In this study, the selection aimed to prove that the isolates produce bacteriocins. Based on isolate selection using well diffusion method from the stock isolates, five isolates could form a clear zone around the colony.

A clear zone was visible around the well hole with a clear edge (circular shape) during diffusion testing using the culture on MRS medium. According to Ray (1992), a clear and faded circular shape of the clear zone indicates that the metabolite acting as the bactericidal is acid. On the other hand, other metabolites that are bactericidal in nature, such as bacteriocins, will provide a clear zone with a clear circular shape. *E. coli* was used in this study as indicator bacteria to prove that the metabolites acted as bactericidal were bacteriocins.

Four isolates with the largest inhibition zone were isolates A, B, C, and D. The diameter of the clear zone around the well showed bactericidal properties (kills bacteria) and the diameter of the pseudo-zone showed bacteriostatic properties (inhibits microbial growth). Screening results were compared to the bacteriocin activity of *L. plantarum* in *E. coli* cultures. *L. plantarum* is known to be able to produce bacteriocins called plantaricin (Todorov, 2008).

Table 2. Result of bacteriocin activity screening from bacteria isolated from tofu whey

Isolates	Bacteriocin Activity **mm^2/mL**
A	2849
B	2346
C	2849
D	2592
E	754
L. plantarum	2346

Based on the measurements, the bacteriocin activity of isolates A and C was the highest compared to the other isolates, which was 2849 AU for both, greater than *L. plantarum* which had an activity of 2346 AU (Table 2). Isolate C was then selected for further research, because of its higher number of colonies compared to isolate A (Table 1), and was one of the samples with the highest bacteriocin activity of all the existing isolates (Table 2). To identify the type of bacteria in isolate C, Macrogen carried out 16S rRNA analysis. Based on Table 1, isolate C is a Gram-positive, rod-shaped and non-spore bacteria. These are some of the characteristics of lactic acid bacteria (Rahayu, 2003). From the analysis using 16S rRNA markers, we found that the species of isolate C was *L. fermentum.* Based on the phylogeny tree (figure 1), isolate C was *L. fermentum* and was closely related to *L.ingluviei*. Bacteriocin known to be produced by *L. fermentum* is fermentcin B that has a narrow inhibitory spectrum. This bacteriocin is

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stable at 100^0C for 30 minutes and is stable in the pH range of 3.0 - 8.0. Fermentcin B can lose its antibacterial activity after being treated with a-chymotrypsin, proteinase-K, and amyloglucosidase (Yan and Lee, 1997).

and more difficult nutrient intake due to the limited number of nutrients, as well as the lower pH value of the medium. Low acidity (pH) of the medium can also inhibit growth or kill *L. plantarum* (Madigan *et al.*, 2009).

During the growth phase, the pH of the

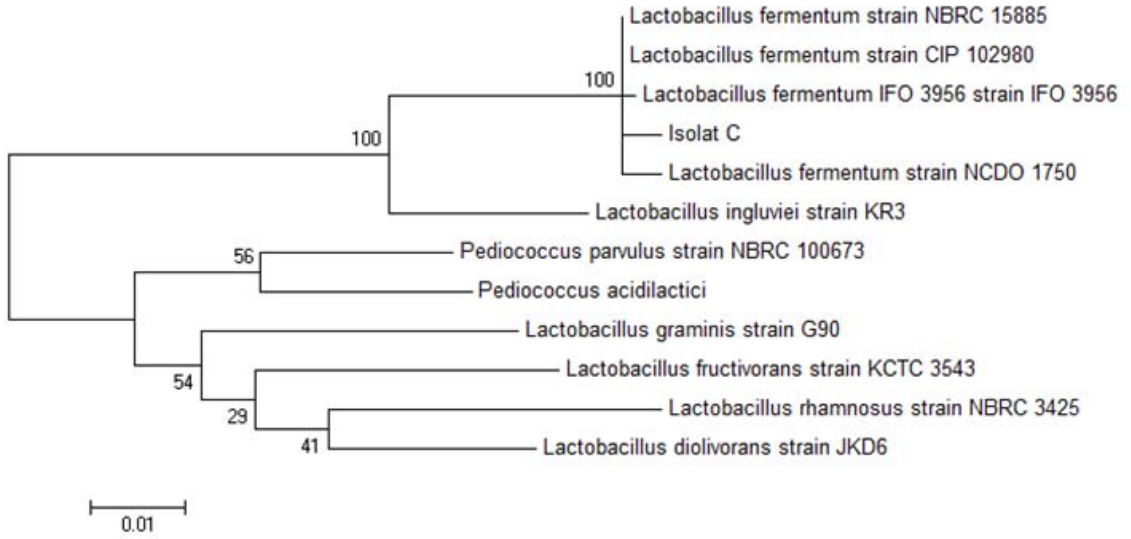

Figure 1. Phylogenetic tree of isolate C which was isolated from whey tofu, made using software MEGA6. Based on these data, concluded that isolate C was *Lactobacillus fermentum*.

Growth Analysis Based on the growth curve, the age of the best inoculum was determined. At the optimum inoculum age, bacteria have the fastest and most active growth rates. The optimum inoculum age is chosen when the bacteria are in the ½ logarithmic phase (Stryer *et al.*, 2002). The optimum inoculum age of *L. plantarum*, *L. fermentum* and *L. mesenteroides* was determined by making growth curves based on the bacteria growth on MRS broth medium. Culture activation is mainly carried out to shorten the lag phase so that the fermentation process can run more quickly. Based on the growth curve in Figure 2, the culture did not go through the adaptation phase, from 0 to 12 hours, *L. plantarum* experienced an exponential growth phase. The growth of *L. plantarum* reached its highest rate at 6 hours with the highest μ value of 0.02 h^{-1}. Bacterial growth enters a stationary phase after 12 hours. The stationary phase lasts from the 12th hour to the 16th hour. The death phase starts to look significant after 16 hours, the cell death rate is -0.001 h^{-1} and the highest cell death rate occurs at 20 to 22 hours reaching -0.02 h^{-1}. Bacterial death can be caused by the denser bacteria in the medium which results in higher competition culture decreased from initial pH of 5.61 to 3.95 after 24 hours. The decrease in pH was due to lactic acid produced by bacteria during the growth phase. This decreasing pH value is one of the causes of bacterial death. The low pH of the medium compared to the cell cytoplasm results in cell death because DNA becomes unstable and the entire metabolic process can be disrupted (Madigan *et al.*, 2009).

The optimum inoculum age is determined from the time during the logarithmic half-phase (Stanburry *et al.*, 1995). By making the growth curve, we found that the logarithmic phase occured for 12 hours, so it can be concluded that the optimum inoculum age of *L. plantarum* on MRSB medium was 6 hours which was also the time of *L. plantarum* reached the highest growth rate of 0.02 h^{-1} with the number of cells $9.2x10^7$ cfu/mL.

The growth curve of *L. fermentum* can be seen in Figure 2. Since 0 hour, the cell number has been increasing, although it was not too high. The growth in the number of cells experienced a spike at 4 to 6 hours and reached the highest growth rate at 6 hours of 0.04 h^{-1}. The logarithmic phase ended at 12 hours, and after that, the cell growth rate decreases. From

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Table 3. Arbitrary value of bacteriocin activity units

Time	Bacteriocin Activity Unit (mm^2/mL)		
	Lactobacillus fermentum	*Leuconostoc mesenteroides*	*Lactobacillus plantarum*
0	0	0	760,39
4	2506,43	2506,43	3341,25
8	1502,68	2939,44	3889,29
12	1546,11	2402,54	3109,03
16	2640,87	2773,79	5523,57

Pathogenic bacteria used were *E. coli* (10^8cfu/mL).

12 to 18 hours, the cell growth experiences a stationary phase because the growth tends to be constant. In the stationary phase, bacteria's growth and death rates are balanced, and there is no significant change in numbers (Todar, 2012).

Figure 2. also shows the correlation between the growth in the number of *L. fermentum* cells with a decrease in the medium's pH. The pH of the medium has decreased from 5.48 to the lowest pH of 4.69. However, at 18 hours, the pH of the medium increased to a pH value of 4.92. It is suspected that the occurrence of dead cells in the culture causes the pH to increase. Lysed cells cause the medium to become more alkaline.

By observing the growth curve, we found that the optimum culture age of the *L. fermentum* inoculum was 6 hours which was half of its logarithmic phase (Stanburry *et al.*, 1995) and it was the time when bacteria experienced the highest growth with a cell number of $8.0x10^7$ cfu/mL.

The growth curve of *L. mesenteroides* shows that these bacteria do not undergo a lag phase or adaptation phase (Figure 2). Since the 0th hour, the growth has entered a logarithmic phase until the 12th hour. In the 6th hour, the growth rate reaches the highest value of 0.02 h^{-1}. After 12 hours, the bacteria experience a decrease in the number of cells. The cell death rate becomes very high when entering the 16th hour, with a rate of 0.07 h^{-1}. The denser the number of cells, the higher the production of acids, and the reduced nutrients can cause cell death. A decrease in acidity (pH) occurs throughout the growth phase since the 2nd hour. The pH decrease occurrs from the initial pH of 5.55 and reaches the lowest pH of 4.25 at 16 hours.

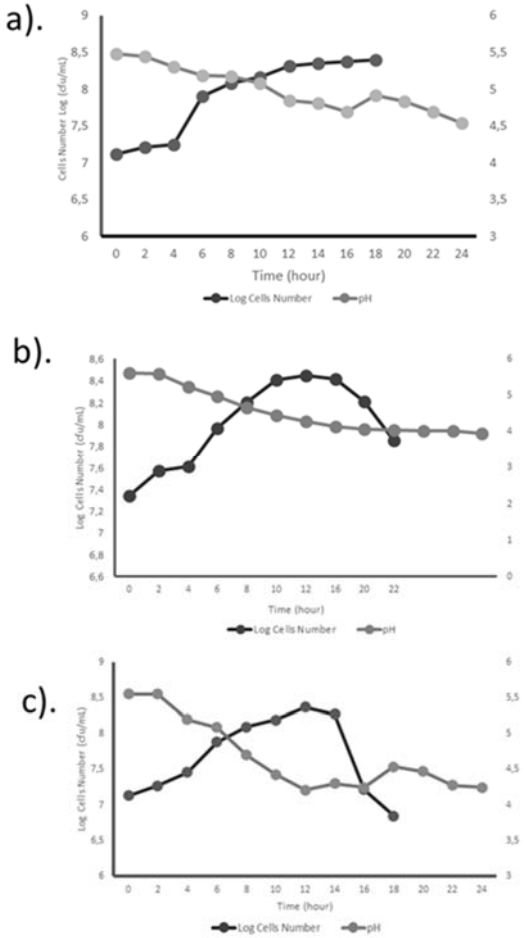

Figure 2. Growth chart of a single culture of a). *L. fermentum*, b). *L. plantarum,* c). *L. mensenteroides* and pH decreased following the time.

Based on growth curve, the logarithmic phase occurs for 12 hours, so it can be concluded that the optimum inoculum age of *L. mesenteroides* on MRSB medium is 6 hours. At this time, *L. mesenteroides* reaches the highest growth rate of 0.02 h^{-1} with cell numbers of $77x10^6$ cfu/mL, which is the half-time of the logarithmic phase (Stanburry *et al.*, 1995).

Bacteriocin Measurement Bacteriocin activity from each single-culture bacterium was tested every 4 hours for 16 hours to see an inhibition zone's formation. Lactic acid bacteria antimicrobial test against pathogenic bacteria was conducted using well diffusion method. Based on the length of incubation time, the highest bacteriocin activity in *L. fermentum* is at 16 hours with the value of 2640.873

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mm^2/mL. *Leu. mesenteroides* shows the highest bacteriocin activity at 8 hours with 2939.444 AU activity value, while *L. plantarum* has the highest activity value of 5523.571 AU at 16 hours (Table 3).

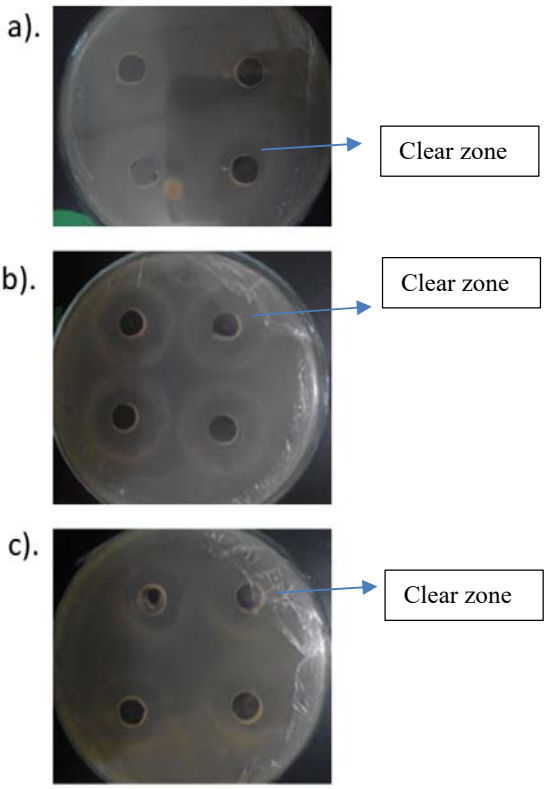

Figure 3. Bacteriocin activity in a). *L. fermentum*, b). *Leu. mesenteroides*, c) *L. plantarum* indicated by the presence of a clear zone around the diffusion well which was inoculated with LAB.

Bacteriocins are synthesized during the exponential phase of cell growth following the classical pattern of protein synthesis. This system is regulated by extra chromosomal plasmid DNA and is influenced by several factors, especially pH. Generally, bacteriocins are synthesized through the ribosomal pathway, while the lantibiotic group is synthesized ribosomally as a prepeptide and then undergoes modification (Chen and Hoover, 2003). Usmiati *et al.*, (2009) reported that the inhibition test result of *Lactobacillus* sp strain SCG 1223 against *E. coli* culture was 623,263 AU. This report shows that *L. plantarum*, *L. fermentum* and *Leu. mesenteroides* have a greater inhibitory ability against *E. coli* than *Lactobacillus* sp. strain SCG 1223.

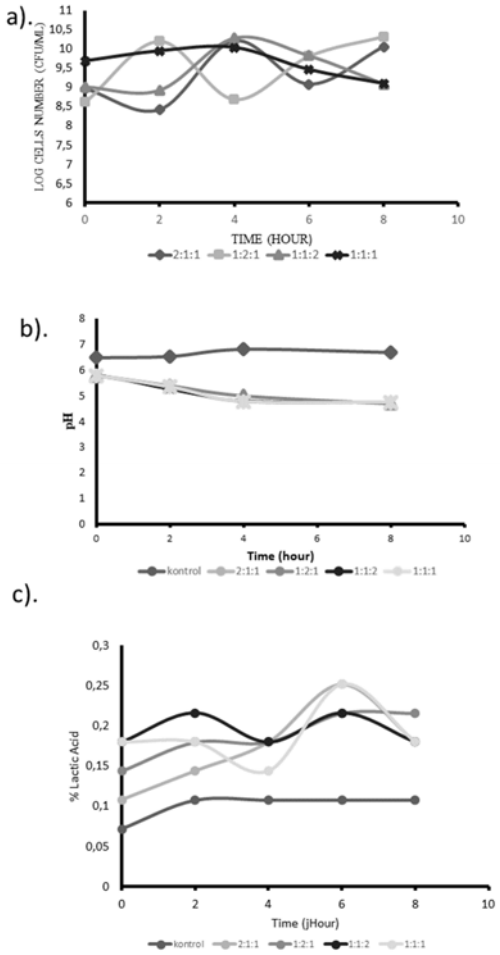

Figure 4.a). Microbial growth pattern of *L. fermentum*: *Leu. mesenteroides*: *L. plantarum* consortium with the ratio of (2: 1: 1); (1: 2: 1); (1: 1: 2); (1: 1: 1) in fermentation process of soy milk medium with initial pH 6.5, incubation temperature 37^{0}C, without agitation; b). Changes in the pH value of the optimized medium during fermentation period for 8 hours in soy milk medium, temperature 37^{0}C, without agitation, c). The increment pattern of lactic acid content produced by microbial consortium *Lactobacillus fermentum*, *Leuconostoc mesenteroides*, and *Lactobacillus plantarum* in the fermentation process of soy milk medium.

Optimization of Fermentation Using Variation in Inoculum Ratio *Lactobacillus fermentum*, *Leu. mesenteroides*, and *L. plantarum* were used as inoculums. Four inoculum ratios were applied for *L. fermentum*:

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Leu. mesenteroides: *L. plantarum*: (2: 1: 1); (1: 2: 1); (1: 1: 2); and (1: 1: 1). Direct optimization was carried out on soy milk media to make tofu using the optimum inoculum age of each bacterium, which was 6 hours (half of its logarithmic phase), and fermentation was carried out for 8 hours.

Pattern of pH Changes and Lactic Acid Increment Lactic acid bacteria were inoculated on soy milk medium until the pH decreased and the isoelectric point reached, hence, coagulating the soy milk protein. The initial pH of soy milk was 6.49. Along with the growth of lactic acid bacteria in the medium, the medium experienced a decrease in pH value.

The pH value decreases during 8 hours of fermentation, and there is no visible difference between each optimization ratio (Figure 5.a). The pH of soy milk medium decreased from 6.49 to 4.7 after 8 hours, which was slightly different with the (2: 1: 1) ratio of 4.73, the (1: 1: 2) ratio of 4.77, and the (1: 1: 1) ratio of 4.75. The decrease in pH was caused by the production of lactic acid and other organic acids by *L. fermentum, L. plantarum,* and *Leu. mesenteroides* (Todar, 2012). Based on Figure 4.c, at the beginning of the measurement, the lactic acid levels are vary. This condition probably happens because the levels of lactic acid formed in each bacteria's stock culture are different. Lactic acid levels should increase over time; the decline that occurs in the middle of the fermentation process is probably due to the fluctuating growth of the bacteria (Figure 4.c). The highest level of lactic acid at the end of the 8-hour fermentation process is obtained by the culture ratio of 1: 2: 1, which is 0.216%. The addition of *Leu. mesenteroides* which is twice the number of other bacteria in ratio of 1: 2: 1, most likely causes the higher content of lactic acid production in that ratio.

Measurement of Bacteriocin Activity of Consortium Bacteria in Soy Milk Data in Table 3 indicates that not all cultures show bacteriocin activity. Apart from cultures with (1: 2: 1), (1: 1: 2), and (1: 1: 1) ratio at 4 hours, and in culture with 1: 2: 1 ratio at 8 hours, there is no clear zone area showing any bacteriocin activity.

Table 3. Bacteriocin activity of *Lactobacillus fermentum*, *Leuconostoc mesenteroides*, and *Lactobacillus plantarum* bacteria in soy milk medium

Inoculum Ratio	Bacteriocin activity (mm^2 / mL) at the hour		
	0	4	8
control	0	0	0
2:1:1	0	0	0
1:2:1	0	495	624
1:1:2	0	1767	0
1:1:1	0	1053	0

Compared to MRS media and single culture, bacteriocin production in soy milk medium is quite limited. Soy milk nutrition is not as complete as MRS media and the possibility of inhibiting mutual activity of cell growth between bacteria, may inhibit bacteriocin activity.

Based on the bacterial growth patterns during optimization (Figure 4.a), at the 4th hour, there is a decrease in the number of bacteria; presumably, this was due to the bacteriocin activity in the culture. At the 8th hour, only the 1: 2: 1 ratio still shows bacteriocin activity of 624 AU/mL. This value is lower than the bacteriocin activity from a single culture. At 8 hours, the culture ratio of 1: 2: 1 shows the highest growth (Figure 5). Based on this information, it is suggested that the optimization culture of *L. fermentum, Leu. mesenteroides,* and *L. plantarum* with a ratio of 1: 2: 1 results in the best growth viability and is still able to show bacteriocin activity at the 8th hour.

Tofu Organoleptic Test

Aroma Organoleptic test results on tofu aroma using 10% inoculum showed that the most preferred ratio of *L. fermentum*: *Leu. mesenteroides*: *L. plantarum* was 1: 2: 1 (sample C) with an average value of 3.75. The resulting tofu is preferred over the control tofu, which has an average score of 3.55. Meanwhile, the average from the ratio (2: 1: 1); (1: 1: 2); (1: 1: 1) of *L. fermentum*: *Leu. mesenteroides*: *L. plantarum* was 3.7; 3.7; and 3.5 respectively.

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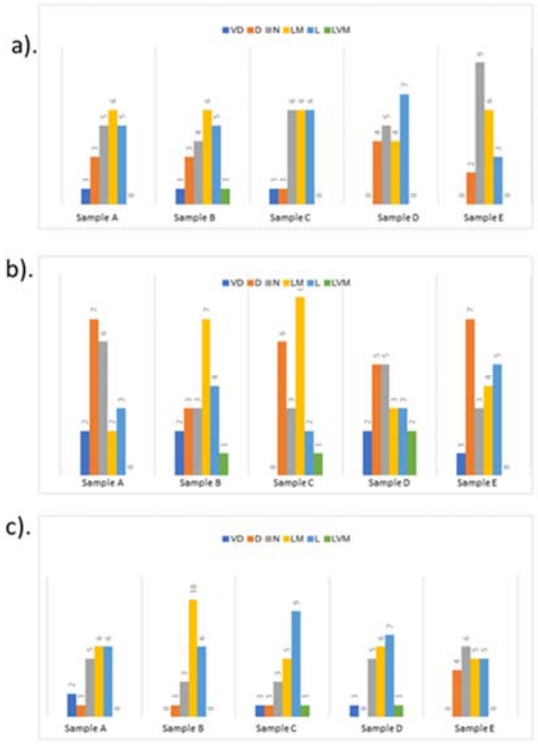

Figure 5. Organoleptic test results of tofu: a). aroma; b). taste, c). texture using *Lactobacillus fermentum*: *Leuconostoc mesenteroides*: *Lactobacillus plantarum* inoculum in ratio of 2: 1: 1 (sample B); 1: 2: 1 (sample C); 1: 1: 2 (sample D); 1: 1: 1 (sample E) and control (sample A). Information: VD = Very Dislike, D = Dislike, N = Neutral, LM = Like Moderately, L = Like, LVM = Like very much.

Texture After pressing and steaming, the tofu was used as a test material for the panelists. The organoleptic test results showed that the most preferred tofu texture was sample C, which used the ratio 1: 2: 1 with 4.15 of preference score. Samples B and D with ratio of 2: 1: 1 and 1: 1: 1 gained the same score of 4.05. Meanwhile, known control and sample E (ratio 1: 1: 1) gained 3.65 and 3.55 score respectively.

Taste The organoleptic test showed that the highest average score for tofu was in sample B, which had 2:1:1 ratio for the comparison of *L. fermentum*: *Leu. mesenteroides*: *L. plantarum* with an average value of 3.55. In terms of taste, fermented tofu, which used whey tofu as coagulant was more preferable to control tofu,. The mean score for control was 2.85. Panelists preferred fermented tofu which was tastier than the control tofu which tasted much more acidic. The average taste score for (1: 2: 1); (1: 1: 2); (1: 1: 1) ratio was 3.45; 3.3; and 3.25 respectively.

Discussion

Bacteriocin activity is measured by calculating the area of inhibition zone minus the well area divided by the volume of the sample inserted into the well expressed by AU. One AU is the area of inhibition per unit volume of the tested bacteriocin sample (mm^2/mL) (Tagg and McGiven, 1971). Bacteriocin activity is expressed as Arbitrary Units per mL (AU/mL) of the culture medium (Simonova and Laukova, 2007). The bacteriocin inhibition zone is associated with a single hit inactivation. One bacteriocin molecule can kill an indicator of the bacterial cell (Ray, 1996). Repetition was carried out for screening process using different well diameters (7 mm and 9 mm in diameter) and sample volumes of 50µL, 100µL, and 150µL, so that clear zones with different diameters were seen. The next test used a well with a diameter of 9 mm with a volume of 100µL.

From Figure 4.a, we can see the growth pattern of each inoculum ratio. Each ratio shows a pattern which is different from one another. The composition of other bacteria influences the pattern in each ratio. The results indicate that mixed cultures slow down the bacteria's growth compared to the growth of single culture because each lactic acid bacteria can inhibit another in mixed culture. According to Gonzalez *et al.* (1994), plantaricin produced by *L. plantarum* can inhibit bacteria from the genus *Leuconostoc*. Meanwhile, the mesentericin produced by *Leu. mesenteroides* can inhibit bacterial growth from the genus *Lactobacillus* (Guyonnet *et al.*, 2000).

When there is cell growth, there is also a decrease in pH and an increase in antimicrobial activity, thereby inhibiting other bacteria's growth in culture. Then after experiencing a reduction in the number of cells, the bacteria re-adjust to the environment and experience growth again. The use of soy milk as the fermentation medium also reduced bacterial cells' growth compared to use standard media such as MRS.

Based on SNI No. 01-3142-1992 revision of SII 0270-80 (SNI, 1992), the parameters that determine the quality of tofu include smell and

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taste, color, and appearance. Based on these standards, good quality tofu has a typical smell and taste. This means that no unpleasant tofu smell is desired. The organoleptic test results showed that the three types of tofu had an unpleasant odor that did not differ during storage.

Judging from the sour flavor parameter, it turned out that control tofu had higher acid flavor than tofu with LAB culture clumping with various ratios. According to SNI (1992), qualified tofu is white or clean yellow. The same thing is stated by Cai and Chang (1998), that tofu desired by consumers is white or yellow in color with very low intensity (pale). From organoleptic test results, the three types of tofus had white color that tended to be slightly yellowish-white. Tofu color resulted from fermentation of all LAB ratios was not significantly different during storage. However, the intensity of white to slightly white yellowish in all ratios of tofu had increased during storage. After the storage test for seven days, LAB fermented tofu, the tofu's level of compactness tended to not change. This showed that the tofu had not been damaged. Meanwhile, the control tofu stored at room temperature was damaged, was no longer in the form of compact tofu, and smelled very rancid. One of the damages that occurs in tofu is the decrease in the physical properties of tofu from compact to soft. This is caused by the growth of contaminant bacteria in the tofu that uses protein and degrades protein into simple compounds. As a result, the chemical bonds in the protein gel become broken.

According to SNI, the standard quality of tofu is normal in appearance, which is not moldy nor slimy. The appearance of tofu on day 1 and 7 did not show any mucus. The change in appearance in the three types of tofus, which appeared on the 7th day, was the discharge of water from the tofu (syneresis). However, syneresis is not a parameter of tofu damage. One of the tofu damage parameters is that the tofu soaking water, or the water that comes out of the tofu is cloudy during storage (turbidity). Turbidity can be caused by the degradation of the nutritional components of tofu into simple water-soluble molecules, caused by microbial activity that destroys tofu.

Organoleptic tests showed that in terms of aroma, panelists prefer starter compositions with the highest *Leuconostoc* ratio. *L. mesenteroides* is known to produce compounds that can affect the taste of food such as lactic acid, acetic acid, ethanol, CO_2, and other flavor compounds (Pagocic *et al.*, 2016). The texture of tofu liked by the panelists was dense, chewy tofu that did not crumble easily. The interaction between the dissolved negatively-charged proteins and the H^+ ion produced by bacteria can cause protein coagulation in soy milk. At the isoelectric point, positive and negative charges in the solution will produce a neutral charge and reduce protein solubility in soy milk, hence curd is formed (Sidar *et al.*, 2012).

In terms of taste, all LAB fermented tofus were liked evenly by the panelists with only a small difference amongst them, and they were all preferred over control tofu. Meanwhile, the addition of *L. fermentum* from whey tofu to increase durability and acidity was also able to improve the taste of tofu so that the panelists liked it.

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