Changes on microbial growth during Mlanding tempeh (Leucaena leucocephala) over fermentation

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Abstract. Mlanding tempeh is made from Leucaena leucocephala seed fermentation by Rhizopus sp. The traditional process of making Mlanding tempeh consist of boiling the seed, crushing to separate the hull, soaking, boiling, and fermentation with Usar as inoculum. There are Rhizopus sp. and some microbial contaminants in Usar. The objective of the research was to study the change on microbial growth (lactic acid bacteria, mold, and yeast) during Mlanding tempeh over fermentation. The fresh tempeh was obtained from 36 hours fermentation and then continued with fermentation up to 120 hours. The results showed that the pH was increased up to 8.5 at the end of fermentation. The total mold number increased at 36 hours fermentation then stable until 72 hours and decreased at the 84 hours fermentation and undetected at 108 hours fermentation. During 36 hours fermentation, the number of yeasts increased then until 72 hours the number of yeasts was stable. In 84 hours the number of yeast decreased and finally, the yeast undetected at the 96 hours fermentation. The number of lactic acid bacteria increased by 72 hours and then decreased until the end of fermentation.

1. Introduction
Lamtoro tempeh or often referred to as Mlanding tempeh is made from Leucaena leucocephala seed fermentation by Rhizopus sp. The tempeh can be found in several regions in Indonesia such as Pacitan, Wonogiri, and Gunungkidul. Mlanding tempeh is a good source of protein. The protein content is 34.39% (db) [1]. The traditional process of making Mlanding tempeh consists of boiling the seed, crushing to separate the hull, soaking, boiling, and fermentation with Usar as inoculum [2]. Usar is the common name of tempeh inoculum in the form of leaves. The name is derived from Javanese which describes how to use it [3]. In the production of Usar, there is not pay attention to aseptic conditions so that there are many contaminant bacteria and varied microorganisms. One of the dominant molds found in Usar is Rhizopus sp. [4].

Microbial diversity in tempeh fermentation has been widely studied. In tempeh, there are other microbes besides Rhizopus sp. In tempeh obtained from the Netherlands, there are various types of bacteria including Enterobacteriaceae exceeded 105 CFU/g from 67% of samples and lactic acid bacteria exceeded 107 CFU/g from 81% of samples. In addition, it is found above 105 CFU/g yeast from 69% of the sample [5]. Mold, yeast, and lactic acid bacteria (LAB) are found in tempeh made with commercial inoculum ‘Raprima’. During the 48 hours fermentation, there is an increase in the number of the LAB from 105 CFU/g to 109 CFU/g. The number of mold increase by 36 hours fermentation and then decreases until the end of fermentation. Whereas the number of yeast is stable during fermentation [6]. In tempeh produced in the Bogor area, mold, yeast, and lactic acid bacteria are found in tempeh which is fermented for 3 days. It is found that there is an increase in the number of lactic acid bacteria from 1st day fermentation to the 3rd day, from 7.3 x 106 CFU/g to 2.6 x 108 CFU/g [7]. Other research
shows the same results. During tempeh fermentation there is a change in the number of microorganisms. The number of lactic acid bacteria increases from 5 log CFU/g to 8 log CFU/g, from before fermentation to 72 hours fermentation. The mold increase until the end of fermentation, from 4 log CFU/g at before fermentation to 6 log CFU/g at 72 hours fermentation. While the number of yeast increase from 1 log CFU/g at before fermentation to 8 log CFU/g at 48 hours fermentation and then decreases. The high growth of lactic acid bacteria at the beginning of fermentation contributes to the inhibition of Enterobacter growth and bacterial spores [8].

As with soybean tempeh, Mlanding tempeh if left at room temperature will change on the chemical, sensory, and microbiological that are associated with continued fermentation. The tempeh is called ‘Tempe bosok’ or over fermented tempeh. Over fermented tempeh is usually fermented in 2-5 days longer than normal tempeh [9]. ‘Tempe bosok’ is an over fermented tempeh which the mold no longer survive and fermentation continued by bacteria [10]. The ‘Tempe bosok’ is used as a seasoning for making various traditional foods such as ‘sayur lodeh’ and ‘sambel tumpang’ because it has a distinctive flavor.

In the continued fermentation study (extended 3 days) soybean tempeh marketed in Bogor area, at the end of fermentation, the total number of the mold is 8 log CFU/g, the total number of lactic acid bacteria is 8 log CFU/g, and the number of yeast is 8-9 log CFU/g [11]. One of the factors that affect the microbiological changes during over fermentation is the change of the pH of the tempeh. The research on over-fermented Mlanding tempeh (Leucaena leucocephala) has not been widely studied. The research will study the microbiological changes (the number of lactic acid bacteria, mold, and yeast) during Mlanding tempeh over fermentation.

2. Material and Methods

2.1. Material
The Lamtoro seed (Leucaena leucocephala) and Usar are obtained from Mlanding tempeh producer which is located in Wonogiri Regency. The materials used for the microbial analysis were PDA (Potato Dextrose Agar), Tartaric Acid 0.1% for mold enumeration, MEA (Malt Extract Agar) and chloramphenicol for yeast enumeration and MRSA (de Man Rogosa Sharpe Agar) and CaCO3 1% for the lactic acid bacteria enumeration.

2.2. Mlanding tempeh Processing
Four kg Lamtoro seeds were boiled by adding the ash, then drained and crushed to separate the hull. After that, the seeds were soaked for 24 hours by changing the water in the first 15 hours. Then, the Lamtoro seeds were boiled and drained until the seeds were not too wet. The inoculum was added 5% into the seeds, then packaged, and incubated at 27o - 29o C for 36 hours, and continued until 120 hours.

2.3. Microbial Analysis
Enumeration of the mold using Spread Plate Method, yeast using Spread Plate Method [12], and lactic acid bacteria using Pour Plate Method [13].

3. Results and Discussion

3.1. pH
The changes in pH during over fermentation shown in Figure 1. Based on Figure 1, the pH of tempeh during fermentation ranged from 6.10 to 8.57. The curve shows that the pH increase from 0 to 120 hours fermentation. The longer tempeh fermentation time, the pH increased exceeded pH 8 [14]. The increase in pH value is due to the results of protein metabolism by the molds [15]. Increasing pH occurs significantly at 0 to 60 hours, from pH 6.10 to 8.07. Then the pH is relatively stable from the 60 hours to the 120 hours. In Mlanding tempeh fermentation, the pH at 0 to 48 hours fermentation increase, from 5.75 to 6.03 [16]. In this study, the pH of tempeh during fermentation was higher than previous research. The research using Usar as inoculum that contains various kinds of microorganisms, resulting in
different metabolites, for example, significant amounts of ammonia (NH3) affecting alkalinization during Tempe fermentation. As well as the presence of proteolytic enzymes produced by Rhizopus oligosporus causing the degradation of proteins into amino acids, the dissolved nitrogen increases, and the amount of ammonia (NH3) increases significantly so that the pH value also increases [14]. In addition, the increase in pH is also due to the dissolution of water-soluble organic acids produced from complex compounds such as fat, protein, and oligosaccharides [14].

Figure 1. The Change of pH during over fermentation (Different superscripts showed significant differences (p <0.05) between fermentation times)

3.2. Microbial Change
Analysis of microbiological characteristics in Mlanding tempeh (Laucaena leucocephala) aims to determine the number of microbiological changes that occur in Mlanding tempeh with the inoculum Usar during over fermentation. Tempeh is produced by fermentation for 36 hours. Continued fermentation is carried out from the 36 to 120 hours. The results of changes in the microbiological characteristics of Mlanding tempeh during over fermentation shown in Figure 2.

Figure 2. Growth of Lactic Acid Bacteria (LAB), mold, and yeast during Mlanding tempeh over fermentation
Based on Figure 2, at 0 hours (before fermentation), the number of lactic acid bacteria (LAB), mold, and yeast were 5.81, 3.79, and 5.88 log CFU / g respectively. Detection of LAB, mold, and yeast at 0 hours (before fermentation) comes from Usar that use as a traditional inoculum. Usar contains Rhizopus sp and many microorganisms [4]. Besides containing mold, Usar also contains aerobic bacteria, spores, lactobacilli, Enterobacteriaceae and yeast [15].

The number of mold at the beginning of fermentation is 3.79 log CFU / g. Mlanding tempeh is ready to be harvested after fermented for 36 hours with the physical characteristics of a white appearance because it is covered by mycelium, compact texture, and the distinctive aroma of Tempe [2]. At 36 hours fermentation, the number of mold is 5.53 log CFU / g then increase to 5.95 log CFU / g in the 48 hours. Furthermore, the number of mold decrease to 5.55 log CFU / g in the 60 hours, and to 4.95 log CFU / g in the 72 hours. In the 84 hours, there was a significant decrease in the number of molds, which was 1.85 log CFU / g and in the 96 hours, it was 1.51 log CFU / g. Then in the 108 hours no mold was detected. This is caused by the process of cell autolysis and decreased cellular energy because in this phase the nutrients in the medium are exhausted, and cannot metabolize so that the mold cannot survive [17].

The number of yeast at the 0 hour (before fermentation) was high, exceeded 5.88 log CFU / g, this was thought to be due to Usar containing aerobic bacteria, spores, lactobacilli, Enterobacteriaceae and yeast [15]. In addition, the raw materials and the manufacturing environment also influence the microorganism content in tempeh [18]. This number is higher compared to the number of mold that is only 3.79 log CFU / g, while the number of the LAB at the same hour is almost the same as the yeast which is 5.81 log cfu / g. Yeast can grow together with R. oligosporus, and its growth can encourage the growth of mold in tempeh and change the appearance and flavor of tempeh. But yeast growth does not affect the growth of LAB [6].

At the 36 hours to 72 hours the number of yeasts was relatively stable between 6.96 log cfu / g to 7.49 log cfu / g. The shape of the yeast growth curve is similar to the mold growth curve, but the number of yeast colonies is more than the mold colonies. The results of this study are similar to a study on soybean tempeh that showed growth of yeast at the 36 to 72 hours ranged from 7.00 log CFU / g to 7.80 log CFU / g, then decreased. Likewise in this study, at the 84 hours, the number of yeasts decreased to 2.25 log CFU / g and then undetected at 96 hours [8]. From figure 1, the pH during over fermentation increased up to pH 8. This condition is not good for yeast growth.

The number of lactic acid bacteria (LAB) at the 0 hour is 5.81 CFU log / g. At the 36 hours the number of LAB incresed to 8.80 log CFU / g, then increased slowly at 9.86 log CFU / g to 10.39 CFU / g in the 48 hours until the 72 hours . Then it dropped slightly and was relatively stable in the 84 hours until the 120 hours with a number of 9.13 log CFU / g up to 9.71 log CFU / g. The high number of lactic acid bacteria in Tempe fermentation after 36 hours proves that naturally occurring LAB can continue to grow without being disturbed by mold or other contaminant bacteria. LAB can grow during Tempe fermentation of 5-9 log CFU / g [6]. In this study, LAB can grow above 10 log CFU / g, this is predicted to be due to the use of different raw materials, so that the substrate content is also different, and the use of different Usar as inoculum.

In this study LAB in Mlanding tempeh continued to grow until the end of fermentation (120 hours) with a high number, which is above 9 log CFU / g. It is predicted that LAB continues to grow because it is able to utilize nutrients from the results of mold and yeast metabolism [8]. LAB is able to hydrolyze soy protein, but the amount is lower than mold and yeast [19]. Likewise in this study, the highest LAB was found after two days (48 hours) of fermentation, at 60 and 72 hours are 10.22 and 10.39 log CFU / g respectively. In food fermentation, yeast grows synergistically with LAB, for example in the synthesis of vitamins, amino acids, and purines and breaks down complex carbohydrates to support BAL growth . On the other hand, yeast can consume lactic acid produced by LAB. Lactic acid bacteria found in tempeh fermentation, for example, L. plantarum [16]. The presence of LAB during tempeh fermentation contribute to the safety of tempeh. The presence of L. plantarum in tempeh make acidified so that inhibit the growth of B. cereus and L. monocytogenes [19].
4. Conclusions

Tempeh fermentation for 36 hours resulted in an increase in pH until continued fermentation for 120 hours, which was pH to 8.5. The total amount of mold increased during fermentation at 36 hours, from 3.76 log CFU / g to 5.53 log CFU / g. Then until the fermentation continued at 72 hours the number of molds was relatively stable and began to decline in the 84 hours fermentation then it was not detected at the 108 hours. The number of yeast and LAB at the beginning of fermentation is high, namely 5 log CFU / g. During 36 hours of fermentation, the number of yeast increased to 7.08 log CFU / g. Then during continued fermentation until 72 hours, the number of yeast is still stable. Then at the 84 hours, the number of yeast decreased and finally, yeast was not detected at the 96 hours fermentation. The number of lactic acid bacteria increased to 8.80 log CFU / g at 36 hours fermentation. During the continued fermentation the number of LAB continued to increase until the 72 hours which was 10.39 log CFU / g. Then it continued to decline until the end of fermentation at the 120 hours. The number of LAB became 9.13 log CFU / g.

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