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Effect of substrate type and incubation time on the microbial viability of instant starter for premium tempeh

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Abstract: Premium tempeh starter is a tempeh starter containing a mixed inoculum of *Rhizopus oligosporus* and *Saccharomyces cerevisiae*. Previously, premium tempeh starter was made in the form of liquid culture. This study aims to produce premium tempeh starter in powder form with the best type of substrate and incubation time so that it can be used practically. In this study, the effect of substrate type and incubation time on microbial viability of instant premium tempeh starter was studied. The study was arranged in a Completely Randomized Block Design with two factors and three replications. The first factor was the type of substrate: tapioca flour and rice flour, while the second factor was the incubation time at room temperature: 0, 24, 48, 72, 96, and 120 hours. The instant premium tempeh starter was analysed for pH value, water content, number of fungi, yeast, and bacteria. The microbial viability of tempeh starter was indicated by the growth of fungi, yeast, and bacteria during incubation. The data obtained were analysed by analysis of variance and further tested with the Honest Significant Difference (HSD) test at a 5% significance level. The results showed that rice flour and incubation time of 96 hours produced the best premium tempeh instant starter with the number of fungi of 9.02 Log CFU/g, 9.17 Log CFU/g yeast, 7.81 Log CFU/g bacteria, pH 4.2, and 7.75% water content. Tempeh made using the best premium tempeh instant starter has a chemical composition in accordance with the tempeh product standard (SNI 3144:2015).

Keywords: incubation time, *Rhizopus oligosporus, Saccharomyces cerevisiae,* substrate type, tempeh starter

1. Introduction

Tempeh is a type of food often consumed by Indonesian people. The advantage of tempeh as a food ingredient is that it contains high nutrients, especially protein. Tempeh is favored because of its nutritional advantages, especially the high content of vegetable protein, unique texture, and pleasant taste and aroma [1]. Soka et al. (2014) mentioned that tempeh is also beneficial as a source of fiber for human health [2].

Generally, tempeh is made from soybean, which is fermented using a tempeh starter. Tempeh starter is a material with a collection of fungi spores. One type of fungi commonly used in the production of tempeh is *R. oligosporus* [3]. In general, tempeh made with *R. oryzae* starter has the unique characteristics of a soft and watery texture and a slight sour, bitter, and sweet taste [4]. However, in addition to fungi, several types of yeast are also used in tempeh fermentation. One type of yeast that is often found in tempeh fermentation is *S. cerevisiae,* a known source of β-glucan [5, 6]. β-glucan is a polysaccharide that acts as a biological response modifier [7]; antimicrobial agents against microbes such as viruses, fungi, bacteria, fungi, and parasites [8]; and an anticancer immune response enhancer [9]. Meena et al. (2013) in their research found that β-glucan can give fish immunity to various pathogens [10].

Together with bacteria and fungi, yeast contributes to the superiority of tempeh by producing functional metabolites [11].

Therefore, *S. cerevisiae* can be used as an additional starter in making tempeh. Research on making tempeh with the addition of *S. cerevisiae* to the starter mixture with *R. oligosporus* has been carried out by Rizal and Kustyawati (2019), which can produce tempeh containing β-glucan [12]. In addition, the addition of *S. cerevisiae* increased the aroma and masked the unpleasant earthy taste (langu) in tempeh [13]. The addition of *S. cerevisiae* can produce tempeh with quite high antioxidant activity, namely 82.42% and β-glucan of 0.58% [14]. In fact, in tempe gembus that was given the addition of *S. cerevisiae*, β-glucan was produced at a higher concentration, namely 0.69% [15].

Premium tempeh is a type of tempeh made using a mixture of inocula of *R. oligosporus* and *S. cerevisiae*, so that the resulting tempeh contains a fairly high level of β-glucan compounds. In previous studies, these two microbes are used in the form of liquid inocula, so their usage in tempeh production was impractical [12]. The provision of an instant premium tempeh starter in the powder form is to get a premium tempeh starter that can be used more efficiently and practically. Tempeh starter in powder form requires a substrate that acts as a filler, preservative, or storage media for *R. oligosporus* and *S. cerevisiae* to last for a long time. Premium tempeh starter is different from tempeh starter in general which only contains a small amount of *S. cerevisiae* resulting in less β-glucan compared to tempeh produced using premium tempeh starter. Tempeh with premium tempeh starter contains 0.578% β-glucan [16], while tempeh without the addition of *S. cerevisiae* only contains 0.076% β-glucan [12].

Fungi and yeast can grow well on substrates containing a lot of carbohydrates. Carbohydrates are a source of carbon contained in the substrate that plays a role in providing nutrients for the growth of *R. oligosporus* and *S. cerevisiae*. Carbon sources that can be used in tempeh starter is rice flour [17], wheat flour, tapioca flour [18], and other sources of carbohydrates.

Tempeh starter generally uses rice or rice flour as a substrate because it contains 67.68% starch [19]. Cassava or cassava flour (tapioca), with 65.26% starch [19] is also a suitable substrate in tempeh starter. Tempeh starter in the form of microbes *R. oligosporus* and *S cerevisiae* with tapioca or rice

flour substrate in powder form will make for a more practical tempeh starter.

Another factor that can affect the number of microbial cells in a material is incubation time. The number of microbial cells will increase with incubation time [20]. The duration of microbial incubation on a particular substrate will make the microbes use the nutrients on the substrate well until they reach the logarithmic phase due to the presence of sufficient nutrients during incubation so that the number of microbial cells will increase. The number of *R. oligosporus* and *S. cerevisiae* cells in the tempeh starter powder will determine the quality of the resulting tempeh. Therefore, this study was conducted to determine the effect of substrate type and incubation time on the characteristics of instant starter for premium tempeh and identify the best tempeh starter.

2. Materials and methods

2.1. Materials and research methods

The materials used in this research were pure cultures of *R. oligosporus* FNCC 6010 and *S. cerevisiae* FNCC 3012 obtained from the Inter-University Center of Gadjah Mada, Jogjakarta, Fermipan (produced by Societe Industrielle Lesaffre, Prancis), Raprima (PT Aneka Fermentasi Industri, Indonesia), rice flour (Rose Brand, Indonesia), tapioca (Pak Tani Gunung, Indonesia), Potato Dextrose Agar (PDA) medium, Malt Extract Agar (MEA), Nutrient Agar (NA) (Himedia).

This study was arranged in a Completely Randomized Block Design with two treatment factors and three repetitions. The first factor was the type of substrate: tapioca flour and rice flour, while the second factor was the incubation time at room temperature: 0, 24, 48, 72, 96, and 120 hours. The obtained data were then tested for its homogeneity with Bartlet's test, and the additional data were tested with Tukey's test. Data were analyzed with variegated prints to determine if there was a difference between treatments. If the difference was significant, the data were tested further using Honest Significant Difference (HSD) with a level of 5%.

2.2. Preparation of S. cerevisiae *culture*

The preparation of *S. cerevisiae* culture was performed following the method of Rizal et al. (2022) [21]. Pure cultures of *S. cerevisiae* were cultured in a sterile MEA medium using sterile inoculation needles in a petri dish, then incubated for 24-48 hours at a temperature of 28°C. Colonies were harvested by adding 10 mL of sterile distilled water. Then, it was slowly poured into a 50 mL centrifuge tube. The tube was weighed and rotated at 3000 rpm for 10 minutes. The supernatant in the centrifuge tube was removed, and pure culture pellets of *S. cerevisiae* were obtained. The amount of *S. cerevisiae* was measured using a haemocytometer until 10^7 cells/mL were obtained.

2.3. Preparation of R. oligosporus *culture*

The preparation of *R. oligosporus* culture followed the method of Rizal et al. (2022) [21]. Pure *R. oligosporus* was cultured in PDA medium using a sterilized loop needle, then inoculated onto the entire surface of the medium by the scratch method. Then it was incubated for 5-7 days at 30-35°C so that pure *R. oligosporus* was obtained in the form of medium colonies. Colonies of *R. oligosporus* were then harvested by adding 10 mL of sterile distilled water. Next, the spores of *R. oligosporus* were

centrifuged at 3000 rpm for 10 minutes. The supernatant in the centrifuge tube was then removed to obtain the pure culture pellets of *R. oligosporus*. The number of *R. oligosporus* was measured using a haemocytometer until 10^7 spores/mL were obtained.

2.4. Production of premium tempeh starter

Tapioca and rice flour were each sterilized at a temperature of 121°C for 15 minutes. After weighing 300 grams of each tapioca and rice flour, 180 mL of sterile distilled water was added on each type of substrate and homogenized. The mixture was then inoculated with 6 mL of *R. oligosporus* and 6 mL of *S. cerevisiae* containing 10^7 cells/mL, then homogenized. After that, tapioca and rice flour batters were divided into six treatments each to be incubated at 28°C for different durations: 0, 24, 48, 72, 96, and 120 hours. The results were dried in a 37°C oven for 24 hours, then refined with a blender. After that, observations were made on the number of microbes (fungi, yeast, and bacteria), pH value, and water content.

2.5. Analysis of the degree of acidity (pH)

The pH value was measured using a pH meter according to the AOAC (2016) procedure [22]. The pH value was measured at the same temperature. Before measurement, the pH meter was standardized using standard buffers of pH 4 and 7. Measurements were done by rinsing the electrode with distilled water and drying it with a tissue. The sample was put into a 100 mL beaker, then the electrode was immersed in the sample solution and left for about one minute until a stable number was obtained and the value was recorded.

2.6. Analysis of water content

Water content analysis was performed using the gravimetric method [22]. The principle of water content analysis is that the weight lost during heating at a temperature of 105-110ºC is considered as the water content in the sample. The first step was heating a cup in a 105-110ºC oven for 30 minutes, cooling it in a desiccator for 15 minutes, then weighing it (A). Next, 2g of sample was put into a cup and then weighed (B). The cup containing the sample was dried in an oven at 105-110ºC for 6 hours and cooled in a desiccator for 15 minutes and weighed. After that, the drying and cooling process was repeated until it reached constant weight (C).

The water content contained in the tempeh starter can be calculated using the formula:

$$\text{Water content} = \frac{B-C}{B-A} \times 100\%$$

Description:
A: empty cup weight (g)
B: cup weight + initial sample (g)
C: cup weight + dry sample (g)

2.7. Yeast count

Determining the yeast count in the tempeh starter was done following the procedure of Rizal et al. (2021) [16]. Each tempeh starter was analysed by growing the culture on MEA medium. Then, 1g of sample was mixed 9 mL of 0.85% NaCl, homogenized, then diluted in a series from 10^{-1} to 10^{-7}. Then, 1 mL of each of the last three dilutions was taken, and microorganisms were cultivated on MEA medium using the spread plate method. The yeast was then incubated at 30ºC for 24-48 hours.

2.8. Fungi count

The fungi content in the tempeh starter was counted following the procedure of Rizal et al. (2021) [16]. Each tempeh starter was analysed for total fungus by growing the culture on PDA medium. Then, 1g of sample was mixed 9 mL of 0.85% NaCl, homogenized, then diluted in a series from 10^{-1} to 10^{-7}. Then, 1 mL of each of the last three dilutions was taken, and microorganisms were grown using the spread plate method on PDA medium. The fungi incubation was carried out at 32°C for 24-48 hours

2.9. Bacteria count

One gram of sample was dissolved in 9 mL of sterile diluent to obtain a dilution of 10^{-1}. The dilution was continued in the same way up to 10^{-7}. Then, 1 mL of each of the last three dilutions was taken using a pipette and put into a sterilized petri dish. Next, 15 mL of NA was put into each petri dish, and the petri dish was rotated slowly so that the NA medium was evenly distributed. After the medium solidified, the petri dish was incubated for 24-48 hours at 37ºC in an inverted position. The number of colonies that grew was then counted.

2.10. Premium tempeh production

The tempeh production followed the method created by Alvina and Hamdani (2019), which had been modified. It started with weighing 500 grams of soybeans and then soaking them in water with a ratio of 1:3 for 12 hours. The soybeans were cleaned from the epidermis by boiling for 1 hour at ±90°C. The soybeans were then drained and cooled. After cooling, 100 grams of soybeans were inoculated with 2% (w/w) of the best tempeh starter. Soybeans were then wrapped in perforated PE plastic and incubated at 28-30°C for 40 hours.

2.11. Analysis of proximate of tempeh

For tempeh with the best treatment, a proximate analysis was then performed which included water content (gravimetry, AOAC 2016), fat content (Soxhlet extraction method, AOAC 2016), protein content [23], ash content (gravimetric method AOAC 2016) and carbohydrate content using by different method.

3. Results and discussion

3.1. Degree of acidity (pH)

Based on Figure 1, premium tempeh starter with tapioca substrate had a pH of 5.76, while tempeh starter with rice flour substrate had a more acidic pH of 4.70. The microbial growth in rice flour substrate was higher than that of tapioca substrate, so the pH of tempeh starter with rice flour substrate was lower. Higher growth of yeast on rice flour substrates made more yeast cells break down starch into glucose which was then hydrolysed into organic acids, making the pH in the tempeh starter with rice flour substrate lower than with tapioca substrate. This result is supported in research by Kurniawan et al. (2014) that states that the sugar content in the substrate will be reduced because the yeast will change the substrate into alcohol and organic acids, resulting in low pH during the fermentation process [24].

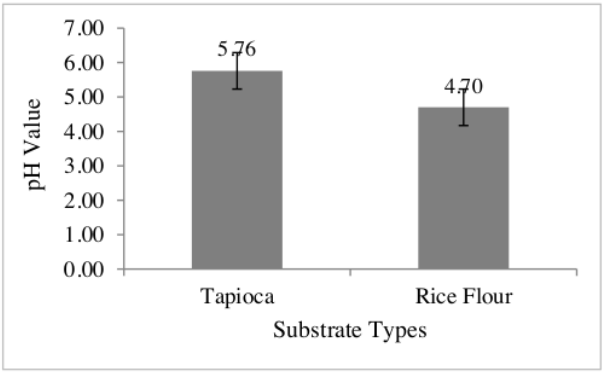

Figure 1. pH values of tempeh starter with different substrate types

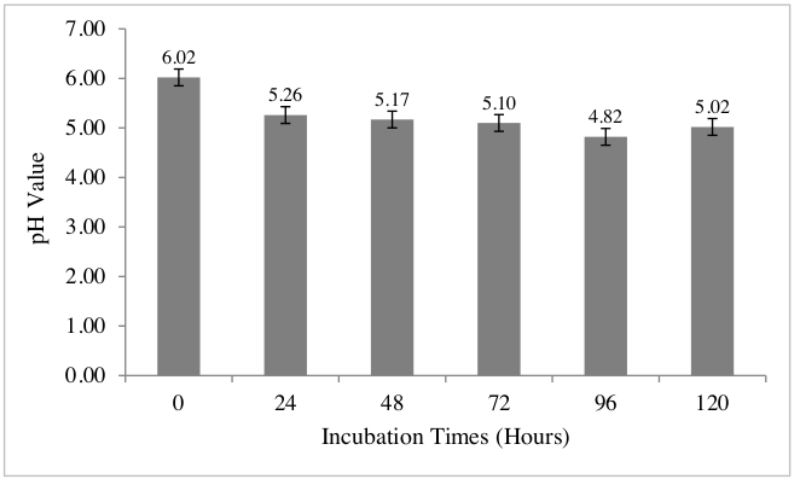

Figure 2. pH values of tempeh starter with different incubation times

Figure 2 shows that the longer the tempeh starter incubation time was, the lower the pH value was. The pH value of the tempeh starter incubated for 0 hours was significantly different from those with

the incubation times of 24, 48, 72, 96, and 120 hours. The decrease in the pH value of tempeh starter as the incubation time decreased was caused by the microbial activity in tempeh starter. As incubation time grew longer, yeast growth increased and yeast cells produced organic acids, lowering the pH value. The results of the pH analysis in this study were in line with the research of Rizal et al. (2020) which showed a decrease in the pH value of tempeh during the fermentation process [18]. Cempaka and Aryantha (2014) confirmed that the decrease in pH of the medium during fermentation could be caused by the formation of primary metabolites such as organic acids by *S. cerevisiae* [25].

3.2. Water content

One factor that can support microbial growth is water. Microbes found in tempeh starter might be affected by the water content in it because water acts as a nutrient for microbial growth. Measuring the water content in tempeh starter was done after the incubation and drying process.

The results (Figure 3) show that the substrate type and incubation time of tempeh starter affected the water content of tempeh starter. The water content ranged from 33.47 to 41.44%. It could be seen that the water content of tempeh starter increased along with the incubation time. This was caused by microbes digesting the substrate and producing water and energy.

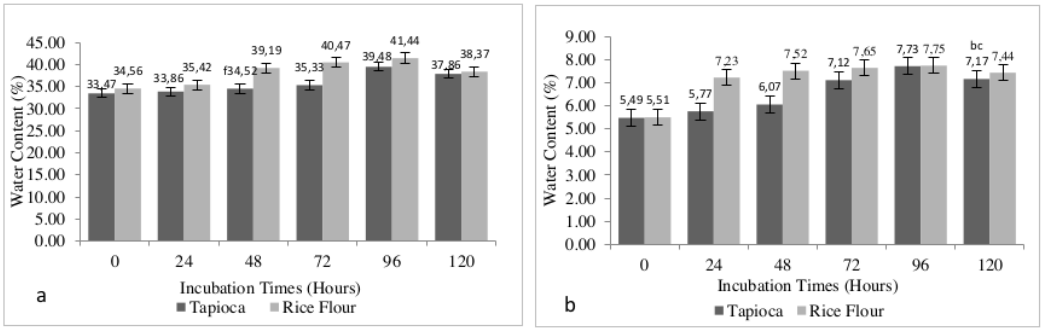

Figure 3. Effect substrate types and incubation times on the water content of instant premium tempeh starter after incubation (a) and after drying (b)

Fermented products using *R. oligosporus* have a short shelf life, so the resulting tempeh starter needed to be dried to reduce its water content. The water content of the tempeh starter was analysed after it was dried in a 37 °C oven for 24 hours. Drying reduces the water content in tempeh starter and makes it difficult for bacteria to grow; thus, the tempeh starter can be stored for a long time. According to SNI 3451:2011, the maximum water content in tapioca products is 14% (Badan Standardisasi Nasional, 2011), so tempeh starters with tapioca and rice flour substrates should have less than 14% water content [26].

The high water content of premium tempeh starter will accelerate the growth of microbes in it, causing rapid loss of nutrients in the substrate so the microbes will quickly experience a death phase. Figure 3 shows that substrate type and incubation time of tempeh starter affected the water content of

dried tempeh starter. The water content from the results ranged from 5.49 to 7.75%, indicating that the tempeh starter's water content after the drying process met the requirements in SNI 3451:2011 [26].

According to Rahman and Mardesci (2015), amylopectin has branched bonds that result in amylopectin having amorphous properties so it is more tenuous, and water will be easier to enter [27]. The higher the amylopectin content in the flour, the more water the starch absorbs. According to Imanningsih (2012), tapioca has a starch content of 65.26% with 8.06% of amylose and 91.94% of amylopectin per % starch, while rice flour has a starch content of 67.68% with 11.78% of amylose and 88.22% of amylopectin per % starch [19]. The amylopectin content in tapioca is higher than in rice flour, so tempeh starter with tapioca substrate was expected to have a higher water content than the one with rice flour substrate. However, Table 6 shows that tempeh starter with rice flour substrate after incubation had a higher water content (38.24%) than the one with tapioca substrate (35.75%).

These results were in line with the results of tempeh starter's water content after drying (Figure 4). Figure 4 shows that dried tempeh starter with rice flour substrate had a higher water content (7.18%) than the one with tapioca substrate (6.56%). In this study, the water content of tempeh starter with rice flour substrate was higher than with tapioca substrate. This could be caused by the initial water content in the substrate being unknown, thus affecting the water content during incubation. Another possible cause was the moisture transfer from the autoclave to rice flour during the sterilization process.

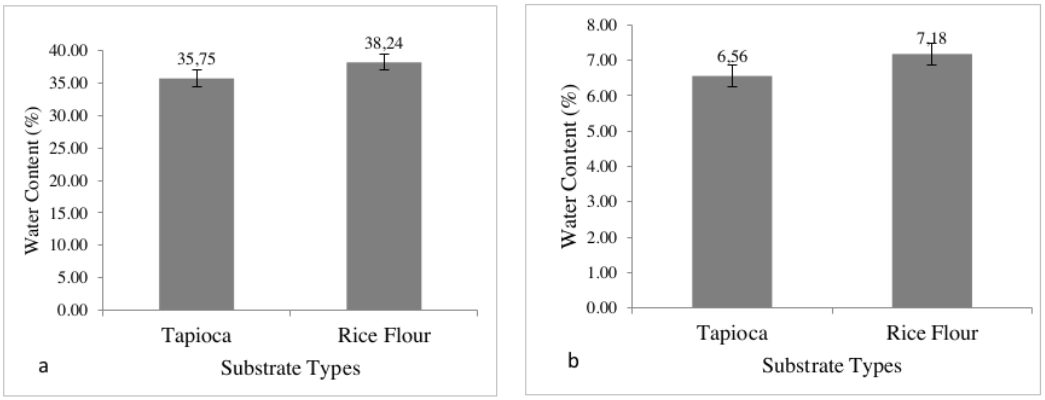

Figure 4. Effect of substrate types on the water content in instant premium tempeh starter after incubation (a) and after drying (b)

Figure 5 shows that the water content of tempeh starter after incubation increased along with the incubation time. The highest water content was found in tempeh starter with 96 hours of incubation time with 40.46% and the lowest was at 0 hours with 34.01%. There was no significant microbial activity in tempeh starter at 0 hours of incubation, so the water content was lower than in other tempeh starters with longer incubation time and more microbial activity.

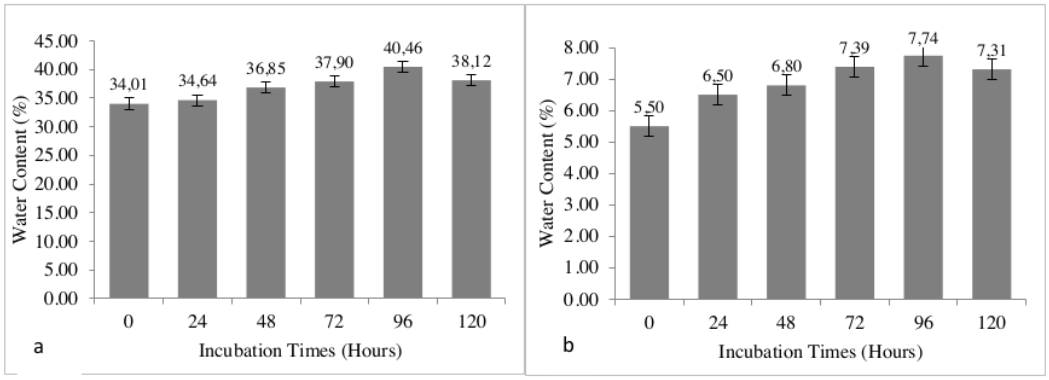

Figure 5. Effect of incubation times on the water content of instant premium tempeh starter after incubation (a) and after drying (b)

This increase in water content might be caused by the absorption of water vapor from the air into the tempeh starter during incubation [28]. The increased water content might also be caused by microbes that produced H_2O during incubation. In the tempeh starter incubated for 120 hours, there was a decrease in water content to 38.12% caused by decreased microbial activity in the tempeh starter at that time.

Figure 5 also shows that the water content of tempeh starter after drying increased along with the incubation time. The highest water content in tempeh starter after drying was found in the starter with 96 hours of incubation at 7.74%, and the lowest was in the one incubated for 0 hours at 5.50%. All data in this study from the water content analysis of tempeh starter after drying met the requirement in SNI 3451:2011 (Badan Standardisasi Nasional, 2011), below 14%.

The water content of tempeh starter after incubation was directly proportional to the water content of tempeh starter after drying, with both increasing along the incubation time. The uniform drying process at 37ºC for 24 hours reduced the water content of tempeh starter to less than 14% and would inhibit bacteria growth, resulting in tempeh starter that could last longer.

3.3. Number of microbes in instant premium tempeh starter

The results showed that the treatment of substrate type and incubation time had a significant effect

on the number of microbes in the premium instant tempeh starter. In this study, the number of fungi, yeast and bacteria in tempeh starter on both substrates used increased from 0 hours of incubation time (Figure 6-11). This showed that fungi, yeast and bacteria could grow well on tapioca and rice flour substrates during incubation. That is in line with Nursiwi et al. (2021) who stated that *R. oligosporus* could grow well on various substrates containing carbohydrates such as rice, tapioca flour, and cassava [17]. The high carbohydrate content of tapioca and rice flour substrates provided adequate nutrition for the fungi during incubation.

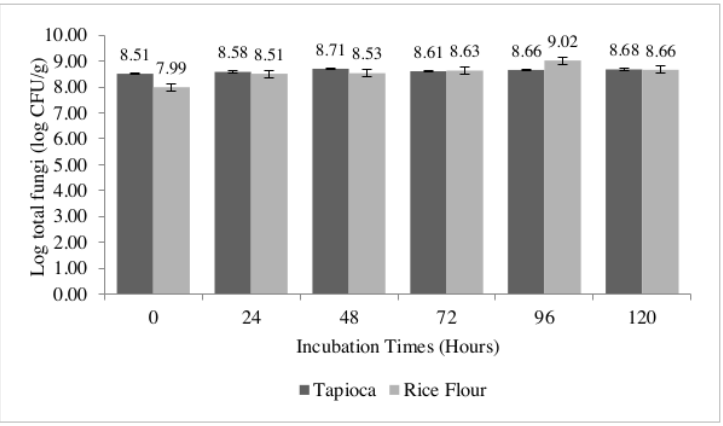

Figure 6. Total fungi of instant premium tempeh starter with different substrate types and incubation times

Figure 6 shows that the highest fungi count was found in tempeh starter with rice flour substrate and incubation time of 96 hours, 9.02 Log CFU/g (1.0×10^9CFU/g). These results are supported by research by Surbakti et al. (2022) showed that mycelial growth in tempeh starter made from rice flour was denser than tapioca [29]. This shows that *R. oligosporus* grew faster on rice flour substrate than tapioca substrate. The nutritional content of rice flour is higher than tapioca. Surbakti et al. (2022) continued that in terms of the carbon source as the main nutrient for the growth of *R. oligosporus*, rice flour has a higher carbohydrate content than tapioca [29]. The amylose content in rice flour substrate was higher than in tapioca, making the rice flour substrate contain more carbon sources for fungi growth. Then, the fungi contained in tempeh starter would produce amylolytic enzymes. These enzymes would break the bonds of amylose and amylopectin into glucose which would then be used as a source of energy for microbial growth [24].

The results of the total fungi count in tempeh starter with different incubation times (Figure 7) revealed an increase from 0 hours with 8.25 Log CFU/g (1.8×10^8CFU/g) to 96 hours with 8.84 Log CFU/g (6.9×10^8CFU/g), the highest number. At 0 hours, the fungi were still adapting to its environment. Then, from 0 to 24 hours, there was an increase in total fungi count because the fungi could utilize nutrients in the substrate optimally until they reached the logarithmic phase. From 48 to 120 hours, the fungi were in the stationary phase, causing no noticeable difference in the total fungi count. The decrease in the total fungi count at a specific time indicated the fungi's death phase due to the nutrients in the substrate beginning to run out. Then, the number of spores in the substrate would get increasingly denser and produce toxic metabolites that stunted fungi growth.

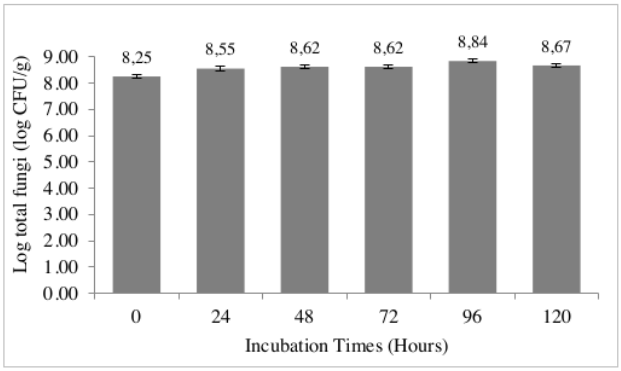

Figure 7. Total fungi count of instant premium tempeh starter with different incubation times

Figure 8 shows that tempeh starter with rice flour substrate had a higher total yeast of 8.75 Log CFU/g (5.6×10^8CFU/g) than the one with tapioca substrate with 8.56 Log CFU/g (3.6×10^8CFU/g). The amount of carbon in tapioca and rice flour substrates can affect the total yeast count in the resulting tempeh starter. That is in line with the research of Rizal et al. (2020) that states the difference in the amount of carbon in the substrate will affect the total yeast count in the resulting tempeh [18]. The higher amount of carbon there is in the substrate, the higher the total yeast. This proves that yeast can utilize the nutrients contained in the substrate.

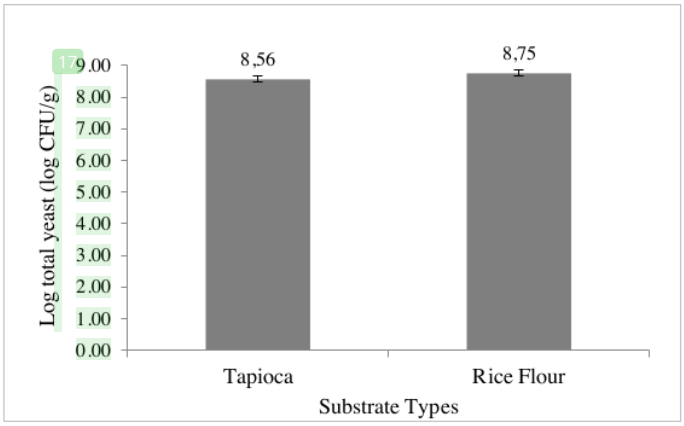

Figure 8. Total yeast of instant premium tempeh starter with different substrate types

The starch content in tapioca and rice flour is broken down by yeast into glucose, which will then be used as a carbon source to meet its nutritional needs for survival [30]. According to Kustyawati et

al. (2013), yeast will produce extracellular enzymes, amylase and protease [31]. During the incubation process, the alpha-amylase enzyme in starch will degrade starch into maltose and maltotriose. The higher the starch content in the substrate is, the more carbon sources are needed to survive and meet the nutritional needs for yeast to survive.

Figure 9 shows that the highest total yeast count in tempeh starter was 8.96 Log CFU/g (9.1×10^8CFU/g), occurring in the starter with 96 hours of incubation. Meanwhile, the lowest total count happened at 0 hours of incubation time with 8.53 Log CFU/g (3.4×10^8CFU/g). There was no significant difference in the total yest count in tempeh starters with incubation times of 0 to 72 hours.

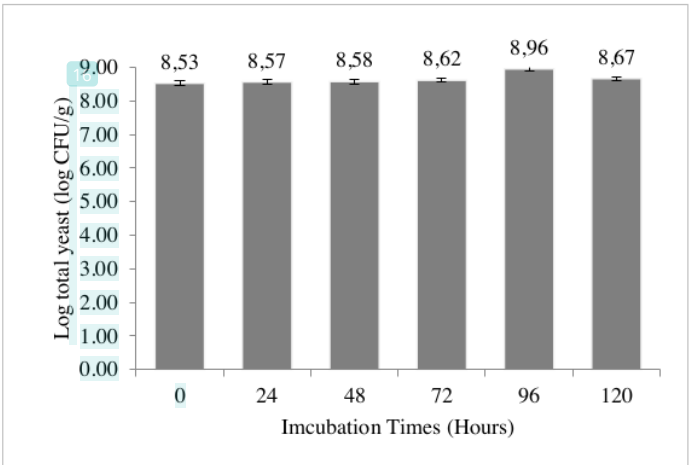

Figure 9. Total yeast count of instant premium tempeh starter with different incubation times

Figure 10 shows that tempeh starter with rice flour substrate had a higher total bacteria count of 7.69 Log CFU/g (4.9×10^7CFU/g) than the starter with tapioca substrate with 7.45 Log CFU/g (2.8×10^7CFU/g). The bacteria that grew on the tempeh starter during incubation were lactic acid bacteria and others that required further identification during incubation (Figure 11). Lactic acid bacteria can grow at acidic pH and inhibit the growth of pathogenic bacteria such as *Escherichia coli*.

According to Imanningsih (2012), tapioca has a starch content of 65.26% with 8.06% of amylose and 91.94% of amylopectin per % starch, while rice flour has a starch content of 67.68% with 11.78% of amylose and 88.22% of amylopectin per % starch [19]. Amylose content in rice flour is higher than in tapioca, so microbial growth was higher in the rice flour substrate due to its nutritional content. This shows that mold, yeast, and bacteria found in premium tempeh starter can grow together utilizing the nutrients available in the substrate.

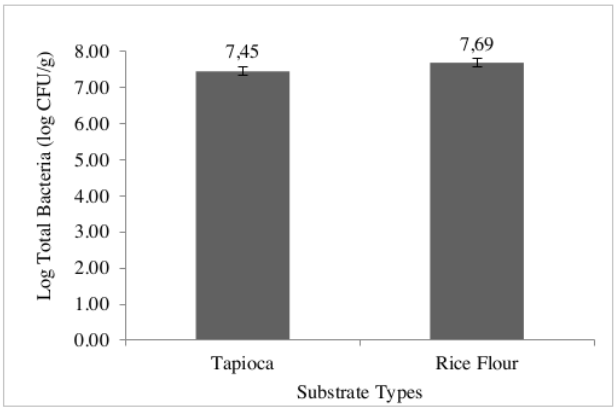

Figure 10. Total bacteria count of instant premium tempeh starter with different substrate types

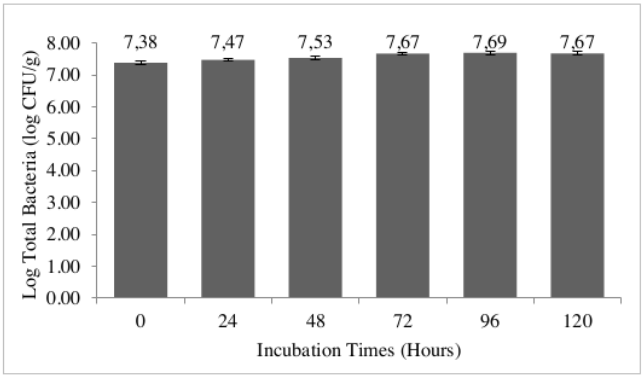

Figure 11. Total bacteria count of instant premium tempeh starter with different incubation times

3.4. The best instant premium tempeh starter

The determination of the best treatment in the manufacture of instant premium tempeh starter was carried out following the weighing index method of De Garmo et al. (1984) on each of the observed parameters [32]. Total fungi count and water content after drying were then tested further using the HSD test at the 5% significance level. Total yeast count, total bacteria count, and the pH value in the combination of substrate type factors and incubation time factors were not significantly different in the 5% HSD follow-up test, so these parameters were not included in determining the best treatment. All treatments fulfilled the SNI 3451:2011 standard provisions of water content in flour products, so it was not the primary determinant. In tempeh production, fungi turn soybeans into tempeh. So, the total fungi count was an important determinant. Recapitulation of treatments of tempeh starter with different substrate types and incubation times can be seen in Table 1.

Table 1. Summary of analysis results for all observed parameters and their weight index values.

Treatment	pH Value	Water Content Before Drying (%)	Water Content After Drying (Max. 14%)	Fungi Count (Log CFU/g)	Yeast Count (Log CFU/g)	Bacteria Count (Log CFU/g)	weighing index
S1T1	6.31±0.49	33.47±0.29	5.49±0.09*	8.51±0.03	8.40±0.14	7.26±0.24	0,40
S1T2	5.83±0.15	33.86±0.02	5.77±0.05*	8.58±0.04*	8.54±0.03	7.40±0.15	0,44
S1T3	5.73±0.26	34.52±0.31	6.07±0.26*	8.71±0.10*	8.50±0.06	7.44±0.18	0,50
S1T4	5.66±0.27	35.33±0.08	7.12±0.05*	8.61±0.07*	8.57±0.13	7.51±0.16	0,45
S1T5	5.44±0.34	39.48±0.19	7.73±0.04*	8.66±0.07*	8.75±0.26	7.58±0.04	0,44
S1T6	5.61±0.19	37.86±0.15	7.17±0.04*	8.68±0.14*	8.59±0.09	7.52±0.06	0,43
S2T1	5.72±0.06	34.56±0.15	5.51±0.14*	7.99±0.35	8.66±0.08	7.50±0.07	0,42
S2T2	4.69±0.06	35.42±0.13	7.23±0.18*	8.51±0.14	8.61±0.32	7.54±0.12	0,44
S2T3	4.62±0.01	39.19±0.32	7.52±0.12*	8.53±0.10	8.65±0.08	7.63±0.14	0,41
S2T4	4.53±0.04	40.47±0.18	7.65±0.03*	8.63±0.17*	8.67±0.35	7.83±0.23	0,49
S2T5	**4.20**±0.06	**41.44**±0.39	**7.75**±0.03*	**9.02**±0.24*	**9.17**±0.22	**7.81**±0.16	0,73
S2T6	4.43±0.05	38.37±0.08	7.44±0.19*	8.66±0.09*	8.76±0.21	7.82±0.38	0,57

Description:
S1 = Tapioca substrate
S2 = Rice flour substrate
T1 = Incubation time 0 hours
T2 = Incubation time 24 hours
T3 = Incubation time 48 hours
T3 = Incubation time 48 hours
T5 = Incubation time 96 hours
T5 = Incubation time 96 hours

Table 1 reveals that the best treatment of tempeh starter with highest weighing index is the S2T5 treatment with the highest total fungi count of 9.02 Log CFU/g (1.0×10^9CFU/g) and a total yeast count of 9.17 Log CFU/g (1.5×10^9CFU/g). The S2T5 treatment was a tempeh starter made from rice flour as a substrate with an incubation time of 96 hours.

3.5. Characteristics of tempeh made by the best instant premium tempeh starter

The characteristics of tempeh produced using the best instant premium tempeh starter (with rice flour substrate and 96 hours of incubation) were white on the entire surface with have a slight grey tinge, compact in texture, with the distinct aroma and taste of tempeh. The white appearance on the tempeh surface indicated that the white mycelia grew evenly. The slightly grey tinge as seen on Figure 12 was due to the greyish-black spores produced by *R. oligosporus*. The mycelia in tempeh increased the density of tempeh, resulting in tempeh that was compact in texture with few air pockets. The distinctive aroma of tempeh was caused by the breakdown of components in soybeans into simpler volatile compounds such as ammonia. The resulting tempeh was fried without using any spices. The fried tempeh had the distinctive tempeh taste caused by the fermentation process of carbohydrates, proteins, and fats in soybeans. Tempeh made from the best instant premium tempeh starter can be seen

393 on Figure 12.

394 **Figure 12.** Tempeh produced with the best instant premium tempeh starter
395
396 The chemical analysis of tempeh produced using the best instant premium tempeh starter can be
397 seen in Table 2. The chemical content of tempeh produced in this study was then compared with tempeh
398 research results by Rizal et al. (2022) which used a liquid starter containing 1% *R. oligosporus* and
399 1% *S. cerevisiae* and tempeh produced using Raprima starter [21].
400 Table 2 shows that the water content of tempeh made with the best instant premium starter not
401 only met the requirement (SNI 3144:2015) but was also lower than that of tempeh produced using
402 liquid starter and Raprima starter. According to Astawan et al. (2013), the water content in tempeh is
403 influenced by the growth of fungi on tempeh [33].
404 The ash content of the tempeh produced using the best instant premium starter was higher than
405 that of tempeh produced using liquid starter and Raprima starter. According to Rizal et al. (2022), the
406 ash content in tempeh is due to the formation of vitamin B_{12} [14]. The presence of *S cerevisiae* can
407 increase the ash content because *S. cerevisiae* is a yeast that produces vitamin B_{12}. The incubation time
408 for making S2T5 tempeh starter caused an increase in the total yeast count so that when inoculated on
409 soybeans in making tempeh, the resulting tempeh had more yeast than tempeh made with liquid starter
410 with direct inoculation did. Therefore, the vitamin B_{12} content in tempeh inoculated using the best
411 instant premium tempeh starter (S2T5) was higher than in tempeh with other starters.
412
413 **Table 2.** The results of the proximate analysis of tempeh produced with the best instant premium
414 tempeh starter (S2T5)
415

Component (%)	Tempeh with the best starter[1] (S2T5)	Tempeh with liquid starter[2]	Tempeh with Raprima starter[3]	Standard of Tempeh (SNI soybean tempeh (3144:2015)
Water content	62.458	64.44	65.435	Maximum 65
Ash content	1.361	1.21	1.210	
Fat content	1.419	8.93	8.765	Minimum 7
Protein content	18.899	16.7	17.110	Minimum 15
Crude fibre	2.492			Maximum 2.5
Carbohydrate content	15.865	8.73	7.480	

Descriptions:

[1] The results of the proximate analysis of tempeh produced using the best instant premium tempeh starter (rice flour substrate and incubation time of 96 hours)

[2] The results of the proximate analysis of tempeh produced using a liquid starter containing 1% *R. oligosporus* and 1% *S. cerevisiae* [21]

[3] The results of the proximate analysis of tempeh produced using Raprima starter [21]

The fat content of tempeh produced using the best starter (S2T5, rice flour substrate and incubation time of 96 hours) was lower than tempeh produced using liquid starter and Raprima starter. That was presumably due to the higher total yeast count in the best starter compared to the liquid and Raprima starter. *Saccharomyces cerevisiae* can reduce fat content in tempeh because *S. cerevisiae* can grow during tempeh fermentation by utilizing carbon and nitrogen sources from soybeans and free fatty acids produced by *R. oligosporus*.

The protein content of tempeh produced by starter S2T5 met the protein standard in SNI 3144:2015 and was higher than the protein content of tempeh produced using liquid starter and Raprima starter. The protein content of tempeh is influenced by *R. oligosporus*, which produces protease enzymes that can break down protein into free amino acids containing N groups to increase the protein content. *Saccharomyces cerevisiae* can increase protein as well because *S. cerevisiae* can produce protease enzymes during its growth [14].

The crude fibre content of tempeh produced using the best tempeh starter (S2T5) fulfilled the quality requirements in SNI 3144:2015, <2.5%. The carbohydrate content of tempeh produced from tempeh using S2T5 starter also was higher than tempeh produced using liquid starter and Raprima starter. The S2T5 starter contained rice flour as a substrate, a source of carbohydrates that microbes can utilize as nutrients for their growth

4. Conclusions

The characteristics of instant premium tempeh starter are influenced by the type of substrate and the incubation time during the manufacturing process. The best characteristic of the instant premium tempeh starter was found in the tempeh starter which made using rice flour with an incubation period of 96 hours with a total fungus of 9.02 Log CFU/g (1.0 x 109CFU/g), total yeast of 9.17 Log CFU/g (1.5×10^{9}CFU/g), total bacteria of 7.81 Log CFU/g (6.5 x 10^{7}CFU/g), pH of 4.2, and water content of 7.75%.

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Conflict of interest

The authors declare no conflict of interest.

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