Substrates and storage time evaluation for preparing tempeh starter from *Rhizopus oryzae* CBS130145

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Abstract: Tempeh is a soy-based product fermented using tempeh starter culture. The starter culture is usually produced traditionally, and therefore has an inconsistent purity and composition. This study therefore aims to determine the viability of *Rhizopus oryzae* CBS130145 grown on various substrates, rice, cassava root, cassava flour, and tapioca flour, then stored for four weeks at 27°C, as well as to determine the texture of the tempeh made using *Rhizopus oryzae* CBS130145, grown on various substrates and commercial inoculum, as a starter. The starter culture was produced using *Rhizopus oryzae* CBS130145 isolated from “usar”, obtained from Wonogiri. According to results, the mold’s viability in each tempeh starter prepared using *Rhizopus oryzae* CBS130145 grown on various substrates, rice, tapioca flour, cassava, as well as cassava flour and stored for four weeks at room temperature, did not differ significantly. In addition, the texture of tempeh produced using all tempeh starter culture did not differ significantly with the commercial starter counterpart. However, further research using lengthened storage time of tempeh starter culture is required to understand the stability of *Rhizopus oryzae* CBS130145 during storage.

1. Introduction

Tempeh is a highly nutritious fermented food product made from soybeans, consumed by most Indonesians, often produced from dehulled, soaked soybean, boiled, drained, cooled, inoculated with starter, usually dominated by *Rhizopus* sp., and left to ferment for 24-48 hours [1,2]. The starter used in producing tempeh is classified as either traditional or commercial. In Indonesia, particularly in Java, traditional starter is often called usar, an inoculum obtained from the leaves of the tempeh wrap. Teak (*Tectona grandis*) leaves are commonly used in Wonogiri region, while senggani (*Melastoma candidum*) is often used in Pacitan region. However, usar is less widely used, compared to commercial starter, due to inconsistent purity and composition.

Meanwhile, RAPRIMA®, widely used as a commercial tempeh starter in Indonesia, contains *Rhizopus oligosporus* [3] as a dominant microorganism. This microorganism produces several enzymes, including lipase, amylase, phytase, and proteolytic enzymes, with the ability to improve tempeh’s nutritional value. However, *Rhizopus oligosporus* has disadvantages, for instance, producing less compact tempeh and sometimes causing an alcoholic odor, as well as only being suitable as the main tempeh fermentation mold in warm areas [4]. In addition to *Rhizopus oligosporus*, some other species of *Rhizopus* sp. Isolates are also dominant microbes in tempeh starter.

*Rhizopus oryzae* is found in the hibiscus leaves used to wrap cooked soybean, thus, these leaves are commonly used as starter [5]. Previous studies isolated *Rhizopus oryzae* CBS130145 from traditional
tempeh starter culture in Wonogiri [6]. Codex Alimentarius has established regional standard for the production of this product, and has deemed *Rhizopus oryzae* as a suitable mold [7]. Generally, tempeh produced using *Rhizopus oryzae* starter has unique characteristics, including soft and watery texture, as well as a slightly sour, bitter, and slightly sweet taste [8]. This study therefore aims to develop instant tempeh starter, using *Rhizopus oryzae* CBS130145 obtained from previous study, in a bid to increase the commercial starter’s diversity.

The substrate is the mold’s source of nutrition for growth, and therefore plays an important role in tempeh starter production, and the mold needs nutrients as a source of energy, including carbon, nitrogen, hydrogen, oxygen. Generally, carbon was obtained from glucose, simple sugars, starches from cereals and roots, and other carbohydrates [9]. Rice and cassava, including cassava flour, have high starch content, and this is required for mold growth [10] [11], while amylase is required to hydrolyze starch into simpler polymers (reducing sugars). In the preparation of tempeh starter culture, *Rhizopus oligosporus* was grown on various substrates, cassava, rice, cowpea, peanut, and soybean. The results showed cassava and rice were the best substrates for mycelia and spore formation, as well as conidia formation [12]. However, other studies used *Rhizopus oryzae* grown on cassava and rice flour as a substrate [13]. This study therefore aims to determine the viability of *Rhizopus oryzae* CBS130145 grown on various substrates, rice, cassava root, cassava flour, as well as tapioca flour, and stored for four weeks at 27°C, and to determine the texture of tempeh made produced using *Rhizopus oryzae* CBS130145 grown on various substrates and commercial inoculum as a starter.

2. Material and Methods

2.1 Culture preparation

*Rhizopus oryzae* CBS130145 was isolated from traditional tempeh starter culture in Wonogiri [6]. Subsequently, the culture was inoculated on Acidified Potato Dextrose Agar (APDA) in a petridish, and incubated at 30°C for 3-7 days, to grow spores [12].

2.2 Inoculum preparation

*Rhizopus oryzae* CBS130145 spores were harvested using 10 ml Tween 80 0.5% with sterile ose. Subsequently, the suspension was obtained and centrifugated at 5000 rpm for 15 minutes to separate the pellets and supernatant. The pellet containing spores was then mixed with about 10ml of sterile water and the number of molds was counted using a hemocytometer. This was followed y using 7 log CFU/ml of mold to produce tempeh starter culture [12].

2.3 Substrates preparation

For this preparation, about 1 kg of rice was boiled (rice and water 1:2) for ±30 minutes, then steamed for ± an hour until cooked. Meanwhile, 1 kg of peeled cassava was grated and steamed for ±30 minutes until cooked. In addition, 1 kg of cassava flour and tapioca flour each were sieved with an 80 mesh filter to remove coarse flour and impurities. Subsequently, all substrates were sterilized with an autoclave at 121°C, for 15 minutes [13].

2.4 Procedure for making starter cultures

For this process, 10 ml of *Rhizopus oryzae* CBS130145 suspension was mixed with 90 ml of sterile aquades in an erlenmeyler flask. The mixture was then inoculated on each substrate (100 ml/kg) placed in sterile tray, then covered with sterile perforated aluminium foil. Subsequently, the tray was incubated at 30°C for 48 hours, and the fermented substrates were dried in cabinet dryer at 50°C, for ±20 hours. The dried fermented substrates were then blended at medium speed for 1 minute and sieved with an 80 mesh sieve. This was followed by tightly sealing the powdered starter culture from each substrate in a plastic bag and storing at room temperature (±27°C). The number of *mold* was then observed 0, 1, 2, and 4 weeks, during the storage [12].
2.5 Determination of viability

After 0, 1, 2, and 4 weeks, 5 g of each culture starter was added to 45 ml of buffer peptone water. Subsequently, 1 ml of each starter mixture was serially diluted using buffer peptone water, and 0.1 ml of each mixture obtained was spread plated in duplicate within APDA and incubated at 30°C for 24 hours, to count the colonies [12].

2.6 Preparation and textural analysis of tempeh

Soybean was boiled for an hour until cooked, cooled to room temperature, dehulled, then soaked in clean water for 18 hours, until a foam and sour smell were obtained. Subsequently, the soybean was washed, boiled again, drained, inoculated with five starter culture types (0.8, 1.2, and 1.6% for each sample starter culture and 0.2% for commercial starter culture), and mixed well. The soybean was then wrapped in oil paper and incubated for two days at room temperature (±27°C). This was followed by testing the texture of tempeh from each substrate, using the Universal Testing Machine[12].

2.7 Statistical analysis

Each parameter was analysed in three replicates, then statistically analyzed using One way analysis of variance (ANOVA), followed by Duncan Multiple Range Test (DMRT), for results with a significance level of 5% . Meanwhile, the data was analyzed using SPSS software program (21.0 version).

3. Result and Discussion

3.1 Viability of Mold

Figure 1 shows the number of the mold during storage at room temperature. The total mold from beginning to the end of storage (four weeks) showed no significant changes in all stater culture types, with the total mold number ranging from 3.6 to 4.9 log CFU/g. This shows each starter culture type made with Rhizopus oryzae CBS130145 and grown in various substrates, was able to produce good quality tempeh. Tempeh fermentation required a minimum mold number of 3-4 log CFU/ml at the beginning of fermentation [14]. Other studies also report no decrease in Rhizopus microsporus var. oligosporus number in tempeh starter culture prepared with various substrates (rice, cassava, cowpea, and soybean), and stored at 25°C for 28 weeks [12].

According to Figure 1, the total number of mold in starter culture prepared with rice and tapioca flour substrates was between 4.7 and 4.9 log CFU/g an was higher, compared to cassava and cassava flour, ranging between 3.6 and 3.9 log CFU/g. This was due to the higher carbohydrate content in rice and tapioca flour (80% and 88%, respectively), compared to cassava of 38% [15]. The high carbohydrate content in rice and tapioca supports mold growth. Carbohydrates (starch) in the substrate are converted into simple sugars by Rhizopus oryzae, and used as carbon and energy sources for growth [16].

3.2 Texture analysis of tempeh

Texture analysis was carried out on tempeh produced using instant starter culture prepared with Rhizopus oryzae CBS130145 grown on various substrates (experimental starter culture), as well as tempeh produced with commercial starter culture (RAPRIMA®). The starter concentrations used with the experimental starter were 0.8, 1.2, and 1.6 % (w/w), while the commercial starter counterpart was 0.2% (w/w).
Table 1: Texture analysis of tempeh made with \textit{Rhizopus oryzae} CBS130145 grown on various substrates and commercial starter culture (RAPRIMA®).

| Type of starter culture | Concentration (%) | \(F_{\text{max}}\) (N) |
|-------------------------|-------------------|-------------------------|
| RAPRIMA® (control)      | 0.2               | 0.62±0.12^a             |
| \textit{Rhizopus oryzae} CBS130145 + Rice | 0.8               | 0.49±0.20^a             |
|                         | 1.2               | 0.65±0.02^a             |
|                         | 1.6               | 0.61±0.12^a             |
| \textit{Rhizopus oryzae} CBS130145 + Cassava | 0.8               | 0.51±0.21^a             |
|                         | 1.2               | 0.43±0.17^a             |
|                         | 1.6               | 0.34±0.20^a             |
| \textit{Rhizopus oryzae} CBS130145 + Cassava Flour | 0.8               | 0.42±0.08^a             |
|                         | 1.2               | 0.57±0.48^ab            |
|                         | 1.6               | 0.41±0.12^a             |
| \textit{Rhizopus oryzae} CBS130145 + Tapioca Flour | 0.8               | 0.53±0.10^a             |
|                         | 1.2               | 0.49±0.14^a             |
|                         | 1.6               | 0.47±0.22^a             |

Note: Different notations in the same column show a significant difference at \(p \leq 0.05\).

Table 1 shows the results of texture (hardness) analysis. According to the table, all the tempeh produced in this study did not differ significantly in texture, and this value ranged between 0.43 and 0.62 N. Similarly, there was no significant difference in appearance (Figure 2). The tempeh’s appearance was the result of the mycelia growth covering the entire product, and this produced a white colour. In addition, the mycelia’s hyphae was kneaded together during the growth to bind the beans, and consequently, construct the tempeh’s compactness [17]. Based on these results, tempeh produced using experimental starter had similar characteristic (texture and appearance) to the commercial starter counterpart.
raprima® 0.2%

|   | Rice | Cassava | Cassava Flour | Tapioca Flour |
|---|------|---------|---------------|---------------|
| 0.8% | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| 1.2% | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| 1.6% | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |

Figure 2: Tempeh made using commercial starter (RAPRIMA®), *Rhizopus oryzae* CBS130145+ rice starter culture, *Rhizopus oryzae* CBS130145+ cassava starter culture, *Rhizopus oryzae* CBS130145+ cassava flour starter culture, and *Rhizopus oryzae* CBS130145+ tapioca flour starter culture.

4. Conclusion

The viability of the mold in all tempeh starter prepared using *Rhizopus oryzae* CBS130145 grown on various substrates, rice, cassava root, cassava flour, and tapioca flour during four weeks storage at room temperature was therefore concluded to not differ significantly. Furthermore, the texture of tempeh produced using experimental starter culture did not differ significantly different with the commercial stater counterpart. Thus, each starter culture produced in this study has the potential to be developed into instant commercial starter for tempeh production. However, further research using lengthened storage time is required to determine *Rhizopus oryzae* CBS130145’s stability during storage.

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